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(54) **Modification of xylanase to improve thermophilicity, alkophilicity and thermostability**

(57) Producing a xylanase enzyme of superior performance in the bleaching of pulp. More specifically, a modified xylanase of Family 11 that shows improved thermophilicity, alkalophilicity, and thermostability as compared to the natural xylanase. The modified xylanases contain any of three types of modifications: (1) changing amino acids 10, 27, and 29 of *Trichoderma reesei* xylanase II or the corresponding amino acids of another Family 11 xylanase, where these amino acids are changed to histidine, methionine, and leucine, respectively; (2) substitution of amino acids in the N-terminal region with amino acids from another xylanase enzyme. In a preferred embodiment, substitution of the natural *Bacillus circulans* or *Trichoderma reesei* xylanase with a short sequence of amino acids from *Thermomonospora fusca* xylanase yielded chimeric xylanases with higher thermophilicity and alkalophilicity; (3) an extension upstream of the N-terminus of up to 10 amino acids. In a preferred embodiment, extension of the N-terminus of the xylanase with the tripeptide glycine-arginine-arginine improved its performance.

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Descripti n

BACKGROUND OF THE INVENTION

5 1. *Field of the invention* The field of the invention is the modification of proteins by protein engineering. In particular, the invention concerns modified xylanase enzymes with improved performance at conditions of high temperature and pH. Xylanase enzymes are used to enhance the bleaching of pulp to make white paper. The invention enables xylanase enzymes to be produced with the benefits of enhanced bleaching associated with Family 11 xylanases, but with activity at higher temperature and pH conditions more suitable to the needs of a pulp mill's operation than xylanases currently available.

10 2. *Brief description of the prior art* Xylanase enzymes have been used commercially since 1991 to enhance the bleaching of pulp to make bright white paper. These enzymes are added to the pulp before the pulp is bleached, and remove a portion of the xylan in the pulp. This action allows the subsequent bleaching chemicals, including chlorine, chlorine dioxide, hydrogen peroxide, oxygen, ozone, and sodium hydroxide, to bleach the pulp more efficiently than in the absence of xylanase treatment. The enhanced efficiency of bleaching has allowed mills to reduce the amount of chlorine-based chemicals to use, which decreases the amount of toxic organochlorine compounds in the mill's effluent, as well as produce whiter pulp or allow the mill to save money on its bleaching chemicals. The commercial use of xylanase enzymes for bleaching has been reviewed by Tolan, et al, Pulp and Paper Canada, December 1995.

15 Xylanase enzymes have been reported from nearly 100 different microbes. The xylanase enzymes are classified into several of the more than 40 families of glycosyl hydrolase enzymes. The glycosyl hydrolase enzymes, which include xylanases, mannanases, amylases, beta-glucanases, cellulases, and other carbohydrases, are classified based on such properties as the sequence of amino acids, the three dimensional structure and the geometry of the catalytic site (Gilkes, et al, (1991) Microbiol. Reviews 55: 303-315).

20 Of particular interest for pulp bleaching applications are the enzymes classified in Family 11. All of these are xylanases and are known as the "Family 11 xylanases". Some publications refer to these synonymously as the Family G xylanases, but we shall use the term Family 11.

25 TABLE 1 lists the Family 11 xylanases known at the present time. Most of them are of molecular mass of about 21,000 Da. Three of the Family 11 xylanases-*Clostridium stercorarium* XynA, *Streptomyces lividans* XynB, and *Thermomonospora fusca* XynA-have a higher molecular mass of 31,000 to 50,000 Da. However, these xylanases have a catalytic core sequence of about 21,000 Da similar to the other Family 11 xylanases. The amino acid sequences of the Family 11 xylanases (or, for the larger enzymes, the catalytic core) show a high degree of similarity (Figure 1). The Family 11 xylanases, which are of bacterial, yeast, or fungal origin, share the same general molecular structure (see Figure 2, of CAMPBELL et al. U.S. Pat.No. 5,405,769).

TABLE 1

Family 11 xylanases	
Microbe	Xylanase
<i>Aspergillus niger</i>	Xyn A
<i>Aspergillus kawachii</i>	Xyn C
<i>Aspergillus tubigensis</i>	Xyn A
<i>Bacillus circulans</i>	Xyn A
<i>Bacillus pumilus</i>	Xyn A
<i>Bacillus subtilis</i>	Xyn A
<i>Cellulomonas fimi</i>	Xyn D
<i>Chainia spp.</i>	Xyn
<i>Clostridium acetobutylicum</i>	Xyn B
<i>Clostridium stercorarium</i>	Xyn A
<i>Fibrobacter succinogenes</i>	Xyn C
<i>Neocallimastix patriciarum</i>	Xyn A
<i>Nocardioopsis dassonvillei</i>	Xyn II
<i>Ruminococcus flavefaciens</i>	Xyn A
<i>Schizophyllum commune</i>	Xyn
<i>Streptomyces lividans</i>	Xyn B
<i>Streptomyces lividans</i>	Xyn C
<i>Streptomyces sp. No. 36a</i>	Xyn
<i>Streptomyces thermoviolaceus</i>	Xyn II
<i>Thermomonospora fusca</i>	Xyn A
<i>Trichoderma harzianum</i>	Xyn
<i>Trichoderma reesei</i>	Xyn I
<i>Trichoderma reesei</i>	Xyn II
<i>Trichoderma viride</i>	Xyn

An enzyme is classified in Family 11 if it possesses the amino acids common to Family 11, including two glutamic acid (E) residues serving as the essential catalytic residues. These E residues are amino acids 86 and 177 by *Trichoderma reesei* xynII numbering. The corresponding location of the key E residues for other Family 11 xylanases is easily determined by aligning the amino acid sequences, a procedure familiar to those skilled in the art. The amino acids common to Family 11 xylanases are indicated in bold type in Figure 1 (Wakarchuk, et al, Protein Science 3:467-475 (1994).

The Family 11 xylanases have several advantages over other xylanases in pulp bleaching applications. Most of the Family 11 xylanases are smaller than xylanases in other families. The small size relative to other xylanases is probably beneficial in penetrating the pulp fibers to release xylan from the pulp and enhance the bleaching. The Family 11 xylanases are also "pure" xylanases in terms of their catalytic activity. Unlike the xylanase enzymes in other families, these enzymes hydrolyze only xylan and do not hydrolyze cellulose. Cellulose hydrolysis damages the pulp and is unacceptable in a commercial mill. Among the Family 11 xylanases, the xylanases made by the wood-rotting fungus *Trichoderma* have been the most widely used in enhancing pulp bleaching. In particular, *Trichoderma reesei* xylanase II (Xyn II), with molecular weight 21,000 and isoelectric point 9.1, has been widely used.

In spite of the advantages of Family 11 xylanases in pulp bleaching, these enzymes have significant drawbacks. The range of temperature and pH that the enzymes exhibits activity on pulp are 45 °C to 55 °C and pH 5.0 to 7.5. A small proportion of mills have operated historically within these ranges. Typically, however, the pulp is at a temperature

of 60 °C to 70 °C and a pH of 10 to 12. In some mills the adjustment of temperature and pH are acceptable and routine. However, in many mills achieving the desired treatment conditions causes severe problems.

Depending upon how the bleaching is carried out, cooling of the pulp to temperatures below 60 °C can decrease the efficiency of bleaching to an unacceptable extent. For example, if a mill is bleaching entirely with chlorine dioxide and has a retention time of less than 20 minutes in the chlorination tower, the minimum temperature for adequate bleaching is 60 °C. If such a mill cannot heat the pulp between the enzyme treatment and the chlorination, which is often the case, then lower temperatures for the enzyme treatment stage are unacceptable.

Sulfuric acid is used to control the pH of the pulp. Depending on the metallurgy of the equipment, the use of sulfuric acid to control the pH can corrode the steel pipes and other equipment. Sulfuric acid is also a safety hazard.

Another minor problem with using these enzymes, and in particular *Trichoderma reesei* xylanase for bleaching applications is the low thermostability. There is the possibility that the warm ambient temperatures in the mills can inactivate the enzymes after several weeks storage. This problem is not as important as the difficulties of adjusting the temperature and pH of the pulp, but must be taken into account by using refrigerated storage or adding stabilizer compounds to the enzyme.

Therefore, the use of xylanase enzymes, particularly Family 11 xylanase enzymes, active at higher pH and temperature ranges than *Trichoderma reesei* Xyn II would be desirable. It would allow mills that operate outside of the active ranges of *Trichoderma* xylanase to be able to carry out xylanase treatment and obtain the benefits associated with the treatment. It would also allow mills to carry out xylanase treatment using less sulfuric acid and cooling water than is currently the case, saving production costs and increasing controllability and storage stability.

Before discussing the approaches that have been taken to improving the properties of xylanase enzymes, it is useful to define the following terms.

Thermophilicity is defined herein as the ability of an enzyme to be active at a high temperature. For example, xylanase #1 has more thermophilicity than xylanase #2 if it is capable of hydrolyzing xylan at a higher temperature than xylanase #2. Thermophilicity relates to enzyme activity in the presence of substrate. In the present invention, the substrate can be pulp xylan or purified xylan.

It is important to specify the substrate for purposes of defining the thermophilicity. Most xylanase enzymes are effective at higher temperatures in the hydrolysis of pure xylan than in the treatment of pulp. This is due to a combination of factors relating to the substrates (i.e. inhibitors present in the pulp) and to the length of time, pH, and other aspects of the procedures used to carry out the tests. Quantitative measures of thermophilicity refer herein to pure xylan substrates unless otherwise indicated.

Thermostability is defined herein as the ability of an enzyme to be stored incubated at a high temperature in the absence of xylan substrate, and then exhibit xylanase activity when returned to standard assay conditions. For example, xylanase #1 is more thermostable than xylanase #2 if it can be held at 70°C for 24 hours and retain all of its activity, while xylanase #2 loses all of its activity after 24 hours at 70 °C. In contrast to thermophilicity, thermostability relates to the enzyme activity remaining after incubation in the absence of xylan substrate.

These two terms are defined explicitly to overcome confusion in the literature, where the two terms are often used synonymously or to denote each other. Their present usage is consistent with Mathrani and Ahring, Appl. Microbiol. Biotechnol. 38:23-27 (1992).

Alkalophilicity is defined herein as the ability of an enzyme to be active at a high (alkaline) pH. For example, xylanase #1 has more alkalophilicity than xylanase #2 if it is capable of hydrolyzing xylan at a higher pH than xylanase #2. Alkalophilicity is analogous to thermophilicity and relates to enzyme activity in the presence of xylan substrate.

For improving xylanase for pulp bleaching applications, the thermophilicity and alkalophilicity are much more important than the thermostability. Most of the work described in the prior art has focussed only on improving the thermostability.

Two generic approaches can be taken to make xylanase enzymes with higher pH and temperature ranges. These are: (1) screening naturally-occurring xylanase enzymes with the desired properties, and (2) using protein engineering to improve the properties of existing xylanase enzymes.

Among naturally occurring xylanases, thermostable enzymes have been isolated from thermophilic microbes, such as *Caldocellum saccharolyticum*, *Thermatoga maritima* and *Thermatoga* sp. strain FjSS3-B.1, all of which grow at 80-100°C (Lüthi et al. 1990; Winterhalter et al. 1995; Simpson et al. 1991). However, all are relatively large in size with high molecular mass of 35-120 kDa (320-1100 residues). Some of these xylanases (*C. saccharolyticum* xylanase A) belong to families other than Family 11 and have both xylanase and cellulase activities (Lüthi et al. 1990). Such cellulase activity is undesirable for pulp bleaching. Furthermore, hyperthermostable xylanases which function normally at extremely high temperatures have low activity at the comparatively lower temperatures for pulp bleaching.

Most of the Family 11 xylanases are effective in pulp bleaching applications at 45 °C to 55 °C. However, Family 11 also includes at least two thermostable xylanases, both of which happen to have a higher molecular mass than the other Family 11 xylanases. These xylanases are *Thermomonospora fusca* xylanase (known as TfxA) of 296 amino acids and 32,000 Da (Irwin et al. (1994) Appl. Environ. Microbiol. 60:763-770; Wilson et al. 1994, WO 95/12668) and

Clostridium stercorarium xylanase A which is of 511 amino acids and 56,000 Da with an optimum temperature of 70°C (Sakka et al. (1993) Biosci. Biotech. Biochem. 57:273-277).

These thermostable xylanase enzymes have some features that are potential problems in pulp bleaching applications. First, the large molecular weight might limit the penetration of the enzymes into the pulp fibers. Second, these enzymes have at least a single copy of a cellulose binding domain (CBD) not present in the other Family 11 xylanases. The CBD, located in the extended C-terminus of TfxA, causes precipitation of the protein and loss of activity in storage.

Therefore, those naturally occurring xylanase enzymes have limitations.

An alternative approach is to carry out protein engineering of a well-known xylanase enzyme. By using protein engineering, specific changes can be made to the protein which might improve a desired property, such as temperature or pH range, without compromising on secondary properties such as protein solubility.

When carrying out protein engineering to modify protein properties, one must select the general method to use and then the specific sites and modifications to make. The general methods include (1) site-specific mutagenesis, (2) random mutagenesis, (3) chimeric modification, (4) dimerization, and (5) glycosylation. Within each of these general methods, there are an enormous number of options of specific modifications to the protein that one can make. The effects of different mutations on enzyme characteristics, including thermophilicity and alkalophilicity, are often unpredictable. Generally, only a tiny fraction of all possible modifications, if any, provide significant benefit. Therefore, setting out to improve the properties of a protein by protein engineering is a difficult venture, and the limited success to date with Family 11 xylanases reflects this. The work with modified xylanases is described as follows.

Site-specific mutagenesis involves the modification of specific amino acids in a protein. The modifications based on site-specific mutagenesis are known as point mutations. Site-specific mutagenesis of Family 11 xylanases has been used to produce xylanase enzymes of slightly improved thermostability. CAMPBELL et al, (U.S. Pat. No. 5,405,769) described one manner of improvement of *Bacillus circulans* xylanase (abbreviated BcX), a xylanase of Family 11, through two types of modifications. These were (i) intramolecular disulfide bonds, and (ii) site-specific mutations at the N-terminus.

CAMPBELL et al, describes how disulfide bonds may be inserted between amino acids #98 and #152, #100 and #148, and #(-1) and #187, according to the amino acid numbering of *Bacillus circulans* xylanase. The disulfide modifications improved the thermostability of the xylanase at 62°C. However, these disulfide-modified enzymes showed no gain in thermophilicity (Wakarchuk et al. (1994) Protein Engineering 7:1379-1386). Therefore, thermostability and thermophilicity are not necessarily coupled.

CAMPBELL et al, also describes three modifications (designated T3G, D4Y(F) and N8Y(F)) near the N-terminus of BcX generated mutant xylanase with thermostability at 57°C, a small increase of 2° C. In the PCT publication WO 94/24270, which is related to CAMPBELL et al, there is a description of a fourth advantageous modification, S22P, for the improvement of BcX. This set of four modifications (designated TS19a in the document) showed a higher thermostability and thermophilicity than BcX. However, certain factors would limit the application of these modifications in Family 11 xylanases other than BcX. These mutations to convert residues-3, 4, 8 and 22 (BcX amino acid numbering) respectively into Glycine, Tyrosine (or Phenylalanine), Tyrosine (or Phenylalanine) and Proline, respectively, are irrelevant to the majority of the Family 11 xylanases, as they already possess these "good" residues (see Figure 1). The best illustration of the inadequacy of these modifications is Xyn II of *Trichoderma reesei*, which possesses all four "good" residues, yet is mediocre in thermophilicity and alkalophilicity.

Random mutagenesis involves the modification of amino acids at random within the entire protein. This method was used to produce a Family 11 xylanase with improved thermostability by ARASE et al ((1993) FEBS Lett. 316:123-127), which described modest improvement of thermostability of a *Bacillus pumilus* xylanase (abbreviated BpX) through modifications at residues-12, 26, 38, 48 and 126 (according to the BpX amino acid numbering). However, ARASE et al did not report any improvement in the thermophilicity or alkalophilicity as a result of their particular modifications. The gain in thermostability by the most improved ARASE et al example in a BpX xylanase was small, only allowing the maintenance of 40% of the residual enzymatic activity after incubation at 57°C for 20 min. For two other BpX xylanases, with the modifications of residues 12 and 26 around the N-terminus, the gain in thermostability represented the maintenance of 1 and 11% residual activity after incubation, respectively. Furthermore, the BpX xylanase with the residue 26 modification has other modifications as well, so the contribution of this sole modification to thermostability, if any, is unclear from ARASE et al.

A chimeric modification involves substituting some of the amino acids of a protein with a sequence of amino acids from another protein. To our knowledge, such an approach has not been carried out with any Family 11 xylanases.

Dimerization involves combining two molecules into a single protein. This technique has been used to link two BcX molecules via an intermolecular disulfide bond (Wakarchuk, et al, Protein Engineering (1994)). The resulting dimeric BcX showed only an insignificant gain in thermostability, much less than BcX with an intramolecular disulfide bond described above.

It is well known that natural glycosylation, the attachment of carbohydrates to a protein, sometimes improves the thermostability of proteins, including in *Trichoderma reesei* xyn II. Synthetic glycosylation has not been used to improve

these properties in a Family 11 xylanase.

No matter which method of protein engineering is used, a key aspect is determining which amino acids to modify, because few choices will improve the properties of the enzyme. This point is illustrated by the work of Sung, et al, Biochem. Cell Biol. 73:253-259 (1995), who modified amino acid #19 in *Trichoderma reesei* xylanase II from asparagine to lysine. This modification decreased the thermophilicity of the enzyme by 3°C.

Therefore, in spite of a large amount of effort with Family 11 xylanases, there has not yet been a modified Family 11 xylanase produced with significantly improved thermophilicity and alkalophilicity. Such an enzyme, and in particular an engineered version of *Trichoderma reesei* xyn II, would have immediate application to the commercial process of producing bleached pulp with decreased requirements for bleaching chemicals while meeting the process conditions of the mills. Such an enzyme would also have potential application in other areas. Some examples of these are as animal feed additives to aid in the digestibility of feedstuffs, where high temperature pelleting makes current enzymes unsuited in many cases; and the processing of wheat and corn for starch production, in which the high temperatures destroy current enzymes.

SUMMARY OF THE INVENTION

The present invention relates to modifying certain specific Family 11 xylanases so as to improve thermophilicity, alkalophilicity, and thermostability. The invention has particular utility in creating enzymes that will allow a pulp mill to obtain the benefits of enhanced bleaching, known to be associated with Family 11 xylanases, but at much higher temperatures and at pH conditions which are more suitable to preferred mill operation parameters, than any xylanases currently available.

The utility of the present invention is specific to Family 11 xylanase enzymes having the following, two essential characteristics:

- (i): The enzyme is made by *Trichoderma*, *Bacillus*, *Aspergillus* or *Streptomyces*
- (ii): The enzyme comprises the amino acid tyrosine or phenylalanine in position 14 by *Trichoderma reesei* xylanase II numbering, or an equivalent position according to the conventional numbering used to designate other xylanases in enzymes of category (i).

For Family 11 xylanases possessing both of these characteristics, either of the following two types of modifications are taught to surprisingly increase thermophilicity, alkalophilicity, and thermostability of the enzyme:

(1) SITE-SPECIFIC MUTAGENESIS : For those selected xylanases with at least 8 amino acid residues in the N-terminus upstream from position 10 (as per *Trichoderma reesei* xylanase II numbering), the modification comprising a substitution of amino acid 10 with another amino acid. A preferred embodiment is to also substitute amino acids 27 and 29 with valine, methionine, isoleucine or leucine, in addition to the essential step of substituting amino acid 10 with a different amino acid. A most preferred embodiment is to substitute histidine, methionine, and leucine, respectively, for the naturally occurring amino acids which are found at positions 10, 27 and 29.

(2) CHIMERIC MODIFICATION : Replacing a sequence of amino acids in the N-terminal region with an equivalently positioned sequence from *Thermomonospora fusca* xylanase A (Tfx) to form a chimeric enzyme. A preferred embodiment extends the chimeric enzyme upstream from the N-terminus with a tripeptide of Glycine-Arginine-Arginine or a sequence of up to 10 amino acids from the N-terminus of *Clostridium acetobutylicum* (CaX).

Surprisingly, the xylanases modified according to the invention have much improved thermophilicity, alkalophilicity, and thermostability over counterpart unmodified enzymes. Some of these modified xylanases have been found to exhibit up to a 28 °C improvement in thermophilicity, and a 2 pH unit improvement of alkalophilicity over the natural xylanase. Furthermore, the ability of some of the modified xylanases to function at 85°C and pH 9 is significantly better than any of the confirmed thermophilic Family 11 xylanases, including TfxA.

The inventors believe that no modification of a Family 11 xylanase has previously been reported with such surprising improvements in the thermophilicity, alkalophilicity, and thermostability of the Family 11 xylanases.

The modified *Bacillus circulans* xylanases of the present invention in particular are active at much higher temperatures than the 60-70°C temperature ranges disclosed for those modified *Bacillus* xylanases illustrated within ARASE et al. (BpX therein) and CAMPBELL et al. (BcX therein).

Furthermore, the modified xylanases of the invention demonstrate surprisingly improved properties for the problem of treatment of pulp. The modified xylanases also exhibit the overall potency in treating pulp that is typical of Family 11 xylanases at optimum conditions, but not observed with other xylanases.

Protein modifications as taught herein have not been previously reported for Family 11 xylanases, and no prior disclosures have suggested that the improvements as taught herein might be able to increase thermophilicity, alkalophilic-

ity, or thermostability.

ARASE, et al ((1993) FEBS Lett. 316:123-127) described modest improvement of thermostability of a *Bacillus pumilus* xylanase (abbreviated BpX) through modifications at residues-12, 26, 38, 48 and 126, according to the BpX amino acid numbering. These correspond to *Trichoderma reesei* xynII residues 11, 26, 38, 48; and 121, respectively, and not residues according to the principles of the present invention. Furthermore, unlike the present inventors, Arase did not report any improvement in the thermophilicity or alkalophilicity as a result of their teachings. The gain in thermostability by the most improved Arase BpX was small, only allowing the maintenance of 40% of the residual enzymatic activity after incubation at 57°C for 20 min. For two other BpX xylanases, with the modifications of residues 12 (11 in xynII) and 26, the gain in thermostability represented the maintenance of 1 and 11% residual activity after storage, respectively. Furthermore, the BpX xylanase with the residue 26 modification has other modifications as well, so the contribution of this sole modification to thermostability, if any, was unclear.

CAMPBELL, et al., as well as the CAMPBELL, et al PCT publication, illustrate four modifications (designated T3G, D4Y(F), N8Y(F), and S22P) that might be made to *Bacillus circulans* xylanase (BcX). These changes correspond to amino acids 12, 13, 17, and 31 by *Trichoderma reesei* xylanase numbering. The amino acids taught by CAMPBELL, et al already exist in *Trichoderma* xylanase and most of the other Family 11 xylanases, and are therefore are deemed essentially irrelevant toward improving the performance of these xylanases.

The CAMPBELL, et al modifications to *Bacillus circulans* xylanase also do not improve enzyme performance nearly as much as the modified *B. circulans* xylanases of the present invention. Examples 6 and 10 show that the modified *Bacillus circulans* xylanases of the present invention have much higher thermophilicity (+14 °C) and alkalophilicity (+1.5 pH unit) than the best of the CAMPBELL, et al xylanases. In addition, at optimum temperature, the modified *Bacillus* xylanases of the present invention have three fold higher activity than that of CAMPBELL, et al.

Amino acids 10, 27, and 29 have not been previously suggested to be important to the performance of the Family 11 xylanase enzymes. Surprisingly, the inventors have shown that modification of these amino acids in *Trichoderma* xyn II to histidine, methionine, and leucine, respectively, significantly increases the thermophilicity, alkalophilicity, and thermostability of the enzyme.

It should be noted that while three of the naturally-occurring Family 11 xylanases have these particular amino acids in these positions. (xylanases produced by *Bacillus pumilis*, *Clostridium stercorarium* (xyn A), and *Thermomonospora fusca*), none of those three xylanases exhibit the combination of desirable characteristics taught to result from following the present invention. There is no reason to expect that the thermophilicity is due to the presence of these three amino acids, and the natural xylanases do not point to these three amino acids as key to the enzyme performance. While the *Clostridium* and *Thermomonospora* xylanases are thermophilic, the *B. pumilis* xylanase is not thermophilic, as it has an optimum temperature below 40°C (Nissen, et al, 1992). In addition to the three common positions mentioned, there are over 75 positions with identical amino acids in the three xylanases. Both of the *Clostridium* and *Thermomonospora* xylanases contain unique cellulose binding domains which have been postulated to confer thermostability, as in the other Families of xylanases (Fontes, et al, 1995, Biochem. J. 307:151-158).

The inventors do not purport to claim any of the three natural Family 11 xylanases that possess the preferred residues in positions 10, 27, and 29. The invention is restricted to those xylanases with tyrosine or phenylalanine in position 14 by *T. reesei* xyn II, or the corresponding position in other xylanases. This excludes the *B. pumilis* xylanase, which has aspartic acid in this position 14. The invention is also restricted to xylanases made by *Trichoderma*, *Streptomyces*, *Aspergillus*, and *Bacillus*. This excludes the *Thermomonospora* and *Clostridium* xylanases.

In addition to these three xylanases, two others in Family 11 have methionine in position 27 and leucine in position 29. These are *Clostridium acetobutylicum* xynB (optimum temperature 43 °C) and *Streptomyces lividans* xynC (optimum temperature 55 °C). Neither of these enzymes, however, are thermophilic and therefore neither suggests the modifications to positions 27 and 29 will be useful.

The specific choices of histidine, methionine, and leucine in positions 10, 27, and 29 improves the stability of *Trichoderma reesei* xynII. Given, this fact, it will now be recognized by those skilled in the art that any of a number of amino acids substituted into position 10 will improve the properties of the enzyme. It will also be recognized that, given the improvements due to methionine and leucine in positions 27 and 29, any of the hydrophobic, medium sized amino acids, including valine, isoleucine, leucine, and methionine will be beneficial in these positions.

The important discovery in this aspect of the invention is that positions 10, 27, and 29 are important for the stability of *Trichoderma reesei* xynII. Based on this teaching, it will be appreciated by those skilled in the art that this modification will be beneficial to certain other Family 11 xylanases, and that Family 11 xylanases must satisfy two conditions to benefit from this modification.

First, the Family 11 xylanase must possess at least eight amino acid residues upstream from position 10. Several Family 11 xylanases have a truncated N-terminus. The modification to position 10 is not relevant to xylanases with a truncated N-terminus.

Second, the Family 11 xylanase must possess the amino acid tyrosine or phenylalanine in position 14 by *Trichoderma reesei* xylanase II numbering, or the corresponding position in other xylanases. The side chain of tyrosine or phe-

nylalanine at position 14 points directly into the active site of the protein. Tyrosine and phenylalanine are of similar size and each has a six member aromatic ring that potentially may participate in a stacking interaction with the xylose ring when bound to a xylan substrate. The presence of other amino acids in this position causes significant changes to the overall structure of the protein. Therefore, only those Family 11 xylanase enzymes with either of these amino acids in position 14 will be suitable for modifications to position 10, while Family 11 xylanases with other residues in the corresponding position will not be amenable to this modification.

Of the known Family 11 xylanases of *Trichoderma*, *Streptomyces*, *Bacillus*, and *Aspergillus*, only *Trichoderma reesei* xyn II, *Trichoderma harzianum* xyn, *Trichoderma viride* xyn, *Streptomyces lividans* xyn B, and *Streptomyces lividans* xyn C meet these two conditions. Therefore, these are the only enzymes presently known that are suitable for the modifications to positions 10, 27, and 29 taught herein.

The chimeric xylanases of the invention have not been previously reported, and there is nothing published to suggest that these particular chimeric xylanases would be beneficial in xylanase performance. Even more surprisingly, in some cases, the thermophilicity and alkalophilicity of the chimeric xylanases is better than that of either of the individual xylanases that comprise the chimeric xylanase. Examples of these enzymes are the modified xylanases NI-BX6 and NI-BX7 in Examples 7 and 11.

The important discovery in this aspect of the invention was that chimeric modification of segments of the N-terminal region of *Trichoderma reesei* xylanase II or *Bacillus circulans* xylanase A with *Thermonospora fusca* xylanase A could increase the thermophilicity, alkalophilicity, and thermostability of the enzymes.

Based on this teaching, it will be recognized by those skilled in the art that chimeric xylanases with improved performance can be formed consisting of the Family 11 xylanases from other *Trichoderma*, *Aspergillus*, *Streptomyces*, and *Bacillus*, strains with *Thermonospora fusca* xyn A provided that one condition is met. The Family 11 xylanase must possess the amino acid tyrosine or phenylalanine in position 14 by *Trichoderma reesei* xylanase II numbering, or the corresponding position in other xylanases. The side chain of tyrosine or phenylalanine in position 14 points directly into the active side of the protein. Tyrosine and phenylalanine are of similar size and their six member aromatic ring can potentially participate in a stacking interaction with the xylose ring when bound to the xylan substrate. *T. fusca* xylanase A has phenylalanine in this position. Only those Family 11 xylanase enzymes with either tyrosine or phenylalanine in position 14 will be suitable for forming chimeric xylanases with *T. fusca* xylanase A. Family 11 xylanases with other residues in the corresponding position will not be amenable to this modification.

Of the known Family 11 xylanases of *Trichoderma*, *Streptomyces*, *Bacillus*, and *Aspergillus*, only *Trichoderma reesei* xyn II, *Trichoderma harzianum* xyn, *Trichoderma viride* xyn, *Trichoderma reesei* xyn I, *Streptomyces lividans* xyn B, *Streptomyces lividans* xyn C, *Bacillus circulans* xyn A, *Bacillus subtilis* xyn A, *Aspergillus niger* xyn A, *Aspergillus kawachii* xyn A, and *Aspergillus tubigensis* xyn A meet this condition. Therefore, these are the only enzymes presently known that are suitable for the chimeric modifications taught herein.

Upstream extension of the protein has never been reported to enhance the performance of the Family 11 xylanase enzyme, nor has it been reported to be beneficial in other enzymes. There is no reason to expect that adding amino acids upstream of the N-terminus would improve the thermophilicity, alkalophilicity, or thermostability.

There is no reason to expect that upstream extension with the three amino acids glycine-arginine-arginine would improve the performance of the enzyme. This set of three amino acids is not found in any natural xylanases. Two of the natural Family 11 xylanases, *Bacillus pumilis* and *Clostridium stercorarium*, have only a single arginine directly upstream of the N-terminus. Both xylanases do not have the other arginine and glycine which are essential to the improved thermophilicity of both modified BcX and TrX. The *Clostridium* enzyme is thermophilic, but the *Bacillus* enzyme is not, so the natural enzymes do not point to this modification.

There is no reason to expect that upstream extension of the N-terminus with a 10 amino acid sequence from *Clostridium acetobutylicum* xyn B (CaX) would improve the thermophilicity of other Family 11 xylanases. CaX has an optimum temperature of only 43 °C, which is no better than the other Family 11 xylanases. Therefore, it is surprising that a sequence of amino acids from this enzyme would improve the thermophilicity of these other xylanases.

The upstream extensions described herein were demonstrated on chimeric xylanases formed by substituting an N-terminal sequence from *T. fusca* xylanase A into xylanase II from *T. reesei* and xylanase A from *B. circulans*. It will be recognized by those skilled in the art that said upstream extension will improve the performance of chimeric xylanases that can be formed by substituting an N-terminal sequence from *T. fusca* xylanase A into a Family 11 xylanase from *Trichoderma*, *Aspergillus*, *Streptomyces*, and *Bacillus*.

In summary, the inventors have developed unique enzymes with desirable properties for commercial applications to treating pulp to improve its bleaching.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the multiple amino acid sequence alignment among several Family 11 xylanases. Each letter represents an amino acid, with the standard amino acid abbreviations used. The divisions into sections are chosen arbitrarily

to fit the typeset of the page and have no relevance to the structure of the proteins. The 1 to 30 amino acid numbering for *Trichoderma reesei* xyn II is indicated. The amino acids common to at least 80% of the Family 11 xylanases listed are indicated in bold. The residues common to all Family 11 xylanases are underlined. For xylanases of *Clostridium stercorarium*, *Streptomyces lividans* (xynB), and *Thermomonospora fusca*, only the catalytic core sequences are presented.

FIG. 2 shows the main chain structures of the fungal *Trichoderma harzianum* xylanase (ThX) and the bacterial *Bacillus circulans* xylanase (BcX).

FIG. 3 shows the synthetic oligonucleotides for the construction of the gene sequence encoding the *Trichoderma* xylanase in the plasmid pTvX(3-190).

FIG. 4 shows the synthetic oligonucleotides for the construction of the gene sequence encoding the *Bacillus circulans* xylanase BcX in the plasmid pXYbc.

FIG. 5 shows the effect of temperature on the enzymatic activity of NI-TX mutant xylanases. Enzymatic activity was normalized to that at 40 °C.

FIG. 6 shows the effect of temperature on the enzymatic activity of NI-BX mutant xylanases. Enzymatic activity was normalized to that at 40°C. The profile of BcX mutant TS19a of Campbell, et al is also presented.

FIG. 7 shows the effect of pH on the enzymatic activity of NI-TX modified *Trichoderma* xylanases at 65°C. The data are normalized to the maximum enzymatic activity.

FIG. 8 shows the effect of pH on the enzymatic activity of NI-BX modified xylanases at 65 °C. The profile of BcX mutant TS19a is also presented. The data are normalized to the maximum enzymatic activity.

FIG. 9 shows the effect of pH on the enzymatic activity of the modified *Bacillus* xylanases at 50 °C. Data on TfxA published by Wilson et al (PCT, 1995) is included. For the purpose of comparison with TfxA, the enzymatic activity of all modified *Bacillus* xylanases was normalized to the pH 8 results.

FIG. 10 shows the thermostability of modified *Trichoderma* xylanases NI-TX1, NI-TX5, NI-TX10, NI-TX11, TvX(3-190) and the natural TrX at 53°C. Enzymatic activity was normalized to that at 0 min of incubation.

FIG. 11 shows the thermostability of chimeric xylanases NI-TX2, NI-TX5, NI-TX8, NI-TX9 and natural TrX at 68°C. The profile of natural TrX at 53 °C was also included for comparison. Enzymatic activity was normalized to that at 0 min of incubation.

FIG. 12 shows the thermostability of NI-BX and BcX at 70°C. Enzymatic activity was normalized to that at 0 min of incubation.

FIG. 13 shows the effect of temperature on the performance of NI-TX xylanase, recombinant and natural TrX in enhancing the bleaching of pulp (tests carried out by logen Corporation).

FIG. 14 shows the effect of temperature on the performance of NI-BX1 and wild-type BcX in enhancing the bleaching of pulp (tests carried out by logen Corporation).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention comprises modified Family 11 xylanase enzymes that exhibit enhanced properties that are important in commercial pulp bleaching applications, namely thermophilicity, alkalophilicity, and thermostability. The modified xylanases exhibit enhanced properties relative to the natural enzymes. The natural enzymes are selected from the group consisting of Family 11 xylanases from *Trichoderma*, *Bacillus*, *Streptomyces*, and *Aspergillus*. The selection of natural xylanases is further restricted to those xylanases with tyrosine or phenylalanine in position 14 corresponding to the amino acid numbering in *Trichoderma reesei* xylanase II, or the equivalent position in other Family 11 xylanases.

The modifications to the selected xylanase comprise either or both of the following, which are described herein by the amino acid numbering of *Trichoderma reesei* xylanase II and apply to the corresponding aligned amino acids of the other selected Family 11 xylanases:

(1) For selected xylanases with at least 8 amino acid residues in the N-terminus upstream from position 10, substitution of amino acid 10 with another amino acid.

(2) Substitution of a sequence of amino acids in the N-terminal region with an equivalently positioned sequence from *Thermomonospora fusca* xylanase A (Tfx) to form a chimeric xylanase, and extending the protein upstream of the N-terminus with a sequence of up to 10 amino acids from another xylanase.

The invention is concerned with modified xylanases and does not claim naturally-occurring xylanases.

In practicing the invention, the starting point is a Family 11 xylanase. An enzyme is classified in Family 11 if it possesses the amino acids common to Family 11, including two glutamic acid (E) residues serving as the essential catalytic residues. These E residues are amino acids 86 and 177 by *Trichoderma reesei* xyn II numbering. The corresponding location of the key E residues for the other Family 11 xylanases is easily determined by aligning the amino acid

sequences, a procedure familiar to those skilled in the art. The amino acids common to the Family 11 xylanases are indicated in bold type in Figure 1. (Wakarchuck, et al, Protein Science 3:467-475 (1994).

The natural Family 11 xylanase used to practice the invention must be among from those Family 11 xylanases made by *Trichoderma*, *Bacillus*, *Streptomyces*, and *Aspergillus*. The enzyme must also have tyrosine or phenylalanine in position 14 by *Trichoderma xylanase II* numbering, or the corresponding position in other xylanases. The amino acid numbering for *Trichoderma reesei* xyn II is shown in Figure 1.

The substitution of amino acid 10 refers to this amino acid of *Trichoderma reesei* xylanase II. This modification is claimed only for those selected xylanase enzymes that possess at least 8 amino acid residues upstream of the position corresponding to amino acid 10 in the N-terminus.

In a preferred embodiment, the selected xylanase for this modification consists of *T. reesei* xyn II, *T. harzianum* xyn, *T. viride* xyn, *S. lividans* xyn B, or *S. lividans* xyn C. In a preferred embodiment, in addition to substitution of amino acid 10 with another amino acid, amino acids 27 and 29 are substituted with methionine, isoleucine, leucine, or valine. As with amino acid 10, amino acids 27 and 29 are identified using the *Trichoderma reesei* xylanase II numbering illustrated in Figure 1.

In a more preferred embodiment, amino acids 10, 27, and 29 are substituted for histidine, methionine, and leucine, respectively. The modified xylanases of this type are identified as NI-TX11, NI-TX12 and NI-TX13 in the Examples.

The chimeric modification of the xylanase consists of removing a sequence of amino acids from the N-terminal region of the selected xylanase and replacing with a sequence of amino acids from the N-terminal region of *Thermomonospora fusca* xylanase A.

As used herein to describe the present invention, the term "N-terminal region" refers to the first 31 amino acids of the *Trichoderma reesei* xylanase II protein closest to the N-terminus. For other Family 11 xylanases, the N-terminal region consists of the amino acids corresponding to the first 31 amino acids of *Trichoderma reesei* xylanase II when the sequences are aligned. The general definition of N-terminal region used in the art is the first 1/3 of the protein closest to the N-terminus. The definition of N-terminal region used in the present invention is consistent with, but necessarily more precise, than the general definition.

The replacement of the amino acids that have been removed is by an amino acid sequence located in the same position relative to the N-terminus of the xylanases from the two microbes. Where the two xylanases have different numbers of amino acids, the replacement sequence of amino acids is at the location that coincides with the original sequence of amino acids when the amino acid sequences of the two enzymes are aligned so as to match as closely as possible. This alignment of amino acids is familiar to those skilled in the art and is illustrated for some of the Family 11 xylanases in Figure 1.

In a preferred embodiment, the sequence of amino acids 10 to 29 from TrX or equivalent aligned amino acid sequence of another Family 11 xylanase is substituted by the corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A. An example of this modified xylanase is identified as NI-TX4 in the Examples.

In a preferred embodiment, the sequence of amino acids 1 to 29 from TrX or equivalent aligned amino acid sequence of another Family 11 xylanase is substituted by the equivalent aligned sequence of amino acids from *Thermomonospora fusca* xylanase A. An example of this modified xylanase is identified as NI-TX3 in the Examples.

In another preferred embodiment, the 1 to 22 amino acid sequence of *Bacillus circulans* xylanase (BcX) is substituted for the 1 to 31 amino acid sequence of TrX. These two sequences are aligned based on the sequences in Figure 1. An example of this modified xylanase is identified as NI-BX2 in the Examples.

In a preferred embodiment, the selected xylanase for this modification consists of *Trichoderma reesei* xyn II, *Trichoderma harzianum* xyn, *Trichoderma viride* xyn, *Streptomyces lividans* xyn B, *Streptomyces lividans* xyn C, *Bacillus circulans* xyn A, *Bacillus subtilis* xyn A, *Aspergillus niger* xyn A, *Aspergillus kawachii* xyn A, or *Aspergillus tubigensis* xyn A.

Upstream extension consists of adding a sequence of up to 10 amino acids to the low molecular mass Family 11 xylanase upstream of the N-terminus. This upstream extension is carried out in combination with a chimeric modification of the selected Family 11 xylanase with *T. fusca* xylanase A described herein.

In a preferred embodiment, a tripeptide comprising glycine-arginine-(arginine or lysine) is added in the upstream extension from the N-terminus of the Family 11 xylanase, and this extension is carried out in combination with a chimeric modification. Examples of modified xylanases with these modifications are NI-TX9 and NI-BX7.

In a preferred embodiment, one to three basic amino acids are added between the N-terminus and a sequence of 5 to 9 amino acids from the Family 11 xylanase *Clostridium acetobutylicum* xynB (CaX), and this extension is carried out in combination with a chimeric modification. Examples of modified xylanases with these modifications are NI-TX8, NI-BX5, and NI-BX6 identified in the Examples.

In a preferred embodiment, a sequence of amino acids ASAR or ASAK are added upstream of the N-terminus and this extension is carried out in combination with a chimeric modification. Examples of modified xylanases with these modifications are NI-TX7, NI-BX3, and NI-BX4.

Another set of preferred embodiments involves using the modified enzymes of the present invention to treat pulp

and improve its bleachability. Said enzyme treatment is carried out at temperatures of 55 °C to 75 °C that are not in the acceptable range for enhancing the bleaching of pulp using natural Family 11 xylanases. In a preferred embodiment, said enzyme treatment is carried out at a pH of 7.5 to 9.0 that is not in the acceptable range for enhancing the bleaching of pulp using natural Family 11 xylanases.

It is well known to those skilled in the art that the xylanase enzymes of the invention might be useful in applications outside of pulp bleaching. For example, these enzymes might be useful as animal feed additives to aid in the digestibility of feedstuffs, where high temperature pelleting makes current enzymes unsuited in many cases. In addition, these enzymes might be useful in the processing of wheat and corn for starch production, in which the high process temperatures destroy current enzymes. It is well known to those skilled in the art that in these or other applications, the modified xylanase enzymes of the invention can be used in the presence of other enzymes, including but not limited to cellulase, mannanase, beta-glucanase, and amylase.

The present invention will be further illustrated by detailed description of the following examples, which are not to be construed as limiting. Modified xylanases according to the Examples are listed in TABLES 2 and 3.

TABLE 2

<i>Trichoderma reesei</i> xylanases	
xylanase	description
natl TrX	natural <i>T. reesei</i> xylanase.
rec TrX	recombinant TrX produced by <i>E. coli</i> , without posttranslational modification.
TvX(3-190)	recombinant TrX with 5 different residues: Ala-1, Ser-2, Gly-4, Phe-9, Thr-65 and Thr-143.
NI-TX1	TvX(3-190) with a mutation Q162H.
NI-TX2	chimeric; the (1-29) sequence of NI-TX1 was substituted by the same region of Tfx and the N-terminus was extended upstream with the tetrapeptide ASHA added to positions (-4) to (-1).
NI-TX3	chimeric; the (1-29) sequence of NI-TX1 was substituted by the same region of Tfx.
NI-TX4	chimeric; the (10-29) sequence of NI-TX1 was substituted by the same region of Tfx.
NI-TX5	chimeric; the TYTN aa sequence in the (26-29) sequence of NI-TX1 was substituted by the same region of Tfx.
NI-TX6	NI-TX1 with mutations Y27M and N29L.
NI-TX7	chimeric; NI-TX3 was extended with the tetrapeptide ASAR in the (-4) to (-1) positions
NI-TX8	chimeric; NI-TX3 was extended upstream in the (-10) to (-1) positions, with (i) the <i>C. acetobutylicum</i> xynB (23-31) sequence from the (-10) to (-2) positions, and (ii) Arg at the (-1) position (Tfx aa numbering).
NI-TX9	chimeric; NI-TX3 was extended upstream with a tripeptide GRR from the (-3) to (-1) positions (Tfx aa numbering).
NI-TX10	NI-TX1 with mutations N10H and N11D.
NI-TX11	NI-TX1 with mutations N10H, Y27M, and N29L.
NI-TX12	NI-TX1 with mutations N10H, N11D, Y27M, and N29L.
NI-TX13	recombinant TrX with mutations N10H, Y27M, and N29L.

TABLE 3

<i>Bacillus circulans</i> xylanases	
xylanase	description
BcX	wild type <i>B. circulans</i> xylanase.
NI-BX1	chimeric; the (1-22) sequence of BcX was substituted by Tfx(1-31) sequence, and the N-terminus was extended with the tetrapeptide ASHA in the -1 to -4 positions. Modification is same as in NI-TX2.
NI-BX2	chimeric; the (1-22) sequence of BcX was substituted by Tfx(1-31) sequence. Modification is same as in NI-TX3.
NI-BX3	chimeric; N-terminus is identical to NI-BX2, but with the N-terminus extended with the tetrapeptide ASAR at the (-4) to (-1) position (Tfx aa numbering). Modification is same as in NI-TX7.
NI-BX4	chimeric; N-terminus is identical to NI-BX2, but with the tetrapeptide ASAK at the (-4) to (-1) positions (Tfx aa numbering).
NI-BX5	chimeric; the N-terminus extension of NI-BX2 was with (i) the <i>C. acetobutylicum</i> xynB (23-31) sequence at the (-10) to (-2) position (Tfx aa numbering), and (ii) Arg at the (-1) position. Modification is same as in NI-TX8.
NI-BX6	chimeric; NI-BX5 with substitution by Arg at the (-2) position and Gly at the (-3) position (Tfx aa numbering).
NI-BX7	chimeric; NI-BX2 was extended upstream with a tripeptide GRR from the (-3) to (-1) positions (Tfx numbering). Modification is same as in NI-TX9.

Examples 1 through 3 hereafter will describe the production and purification of modified xylanases according to the invention. The surprisingly enhanced thermophilicity, alkalophilicity, and thermostability of modified xylanases NI-TX1 through NI-TX11 and NI-BX1 through NI-BX7 are demonstrated using xylan as the substrate in Examples 4 through 13. The performance of xylanase on a xylan substrate is well known to correlate well with that performance which is observed during actual treatment of pulp. A confirmation of surprisingly enhanced performance using modified xylanases according to the invention in the treatment of pulp, before bleaching, is provided for selected modified xylanases in Examples 14 and 15.

EXAMPLE 1

CONSTRUCTION OF THE TRICHODERMA REESEI MODIFIED XYLANASES NI-TX

Basic recombinant DNA methods like plasmid preparation, restriction enzyme digestion, polymerase chain reaction, oligonucleotide phosphorylation, ligation, transformation and DNA hybridization were performed according to well-established protocols familiar to those skilled in the art (Sung et al., 1986) or as recommended by the manufacturer of the enzymes or kit. The buffer for many enzymes have been supplied as part of a kit or reconstituted following the instruction of the manufacturers. Restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase were purchased from New England BioLabs LTD, Mississauga, Ont. GeneAmp PCR reagent kit was purchased from Perkin-Elmer. A precursor plasmid pXYbc, which is a pUC type plasmid with a *Bacillus circulans* xylanase gene inserted, has previously been prepared and published (Sung et al, 1993; Campbell et al. US patent no. 5,405,769 issued on Apr. 11, 1995). A commonly used *E. coli* strain, HB101 (Clontech Lab, Palo Alto, CA) was used as transformation and expression host for all gene construct. Birchwood xylan was purchased from Sigma (St. Louis, Mo). Hydroxybenzoic acid hydrazide (HBAH) was purchased from Aldrich. Oligonucleotides were prepared with an Applied Biosystem DNA synthesizer, model 380B. Xylanase assays have been performed in a covered circulating water bath (Haake type F 4391) with a fluctuation of $\pm 0.1^\circ\text{C}$. Temperature of the water bath was confirmed with a thermocouple.

A. Construction of the precursor plasmid pTvX(3-190).

The precursor plasmid pTvX(3-190) for all subsequent mutations has previously prepared and its construction has already been published (Sung et al, 1995). This plasmid is derived from plasmid pXYbc, through a substitution of the *Bacillus circulans* xylanase gene of the latter by a newly assembled *Trichoderma* xylanase gene. Expression of this

Trichoderma xylanase and other mutant xylanases subsequently described are under the control of the *lac* promoter. The total assembly of the *Trichoderma* xylanase gene required two stages, initially for the (92-190) region, then followed by the (1-92) region. The protocol for the construction of this gene is routine and identical to the standard published procedure for many other genes, using enzymatic phosphorylation of overlapping synthetic oligonucleotides which encodes xylanase, followed by their ligation into an appropriately cut plasmid.

Initially ten overlapping oligonucleotides (XyTv-101 to 110) encoding the TvX(92-190) sequence (Figure 3), were designed with codon usage frequency imitating that of *E. coli*. The Sall and BglII cohesive ends of two terminal oligonucleotides enabled the enzymatic ligation of the ten fragments to the Sall-BglII linearized plasmid pXYbc. Ten oligonucleotides XyTv-101 to XyTv-110 (50 pmol, 1 µL for each) encoding the (92-190) region of *Trichoderma* xylanase was phosphorylated in a mixture containing 10X standard kinase buffer (0.4 µL), 1mM ATP (4 µL), T4 DNA kinase (5 units), and water (3 µL). Phosphorylation reaction was carried out for 1 h at 37°C. The solutions were then combined and heated to 70°C for 10 min. After being cooled slowly to room temperature, the combined solutions were added to a mixture of 4mM ATP (3.5 µL), Sall-BglII linearized plasmid pXYbc (0.1 pmol), and T4 DNA ligase (3.5 µL) and incubated at 12°C for 20 h. Aliquots of the ligation mixture were used to transform *E. coli* HB101 in YT plate (8 g yeast extract, 5 g

bacto-tryptone, 5 g NaCl, 15 g of agar in 1 L of water) containing ampicillin (100 mg/L).

For the preparation of a hybridization probe, one of the oligonucleotide XyTv-110 (10 pmol, 1 µL) was phosphorylated ³²P-ATP (10 pmol, 3 µL) in T4 DNA kinase (1 µL), 10X kinase buffer (1 µL), and water (4 µL) at 37°C for 1 h.

Transformants were selected randomly for hybridization analysis. Colonies were grown on nylon filters on YT plates with ampicillin overnight. They were then denatured with 0.5N NaOH - 1.5M NaCl (10 min) and neutralized with 0.5N Tris-HCl (pH 7.0) - 1.5M NaCl (10 min). After irradiation by UV of 254 nm for 8 min, the filters were washed with 6X SSC - 0.05% TritonX-100 for 30 min. Cell debris was scraped off completely. After another 30 min. in fresh solution, the duplicate filters were transferred individually into separate mixtures of 6X SSC - 1% dextran sulphate - 0.05% TritonX-100 - 1X Denhardt's hybridization fluid. The ³²P-labelled probe was added to the filter. After 16 h at 45°C, the filter was washed twice with 6X SSC - 0.05% TritonX-100 at room temperature for 5 min. and then at 65°C for 30 min. Positively hybridized clones with the intermediate plasmid pBcX.TvX were identified by auto-radiographic analysis.

The above protocol, involving enzymatic phosphorylation of synthetic overlapping oligonucleotides and ligation into a linearized plasmid, has again been used in the assembly of the TX(1-92) region and in the cassette mutagenesis for the subsequent generation of several mutant series NI-TX and NI-BX described in this invention.

For the assembly of the TX(1-92) region to complete the full-length *Trichoderma* gene, the intermediate plasmid pBcX.TvX was linearized by NheI and KpnI endonucleases to release the DNA insert for BcX(1-83). With NheI and KpnI cohesive ends, ten overlapping oligonucleotides (XyTv-1 to -10) encoding the published TvX(3-91) sequence were ligated into the linearized plasmid pBcX.TvX (Figure 3), via the protocol described above. The new plasmid pTvX(3-190) therefore harbored a synthetic TvX(3-190) gene. As compared to the natural TrX, the recombinant TvX(3-190) has five different residues: Ala-1, Ser-2, Gly-4, Phe-9, Thr-65 and Thr-143.

For comparison, a gene encoding the natural TrX has also been assembled in the same manner with the five natural residues. This has also been published (Sung et al, 1995). Expression of this gene in *E. coli* generated a recombinant version of TrX (rec. TrX). As indicated below and in the same report by Sung et al (1995), the performance characteristics of both TvX(3-190) and rec. TrX were identical. However, both *E. coli*-expressed xylanases were worse than the natural TrX (natl TrX) in thermophilicity and thermostability, probably due to the lack of posttranslational modification as in the case of natural TrX.

B. Construction of the plasmid pNI-TX1

All mutant xylanases genes including both NI-TX and NI-BX series described below have been constructed via the method of cassette mutagenesis. The protocol for the cassette mutagenesis was identical to that for gene assembly fully described above. Such cassette mutagenesis involved (i) enzymatic phosphorylation of overlapping synthetic oligonucleotides, (ii) their ligation with the linearized plasmid, (iii) transformation into the *E. coli* HB101 competent cells, (iv) identification of the mutant transformants via hybridization with the labelled oligonucleotide as probe, and (v) confirmation of the mutation through dideoxy nucleotide sequencing.

The mutant NI-TX1 was identical to TvX(3-190) with a single mutation Q162H. The construction of the plasmid pNI-TX1 was through ligation of oligonucleotides TX-162H-1 and TX-162H-2 (shown below) into NsiI/AvrII-linearized plasmid pTvX(3-190) in a cassette mutagenesis.

TX-162H-1

158 159 160 161 162 163 164 165 166
A W A Q H G L T L
5'-TGG GCA CAG CAC GGG TTA ACC
A CGT ACC CGT GTC GTG CCC AAT TGG GAT C-5'
NsiI AvrII

TX-162H-2

C. Construction of the plasmid pNI-TX2

The mutant NI-TX2 is a modified version of NI-TX1, with (i) the (1-29) sequence of the latter substituted by the Tfx(1-29) sequence and (ii) the N-terminus extended upstream with the tetrapeptide ASHA in positions -4 to -1. The plasmid pNI-TX2 was constructed through the ligation of overlapping oligonucleotides Tfx-1, -2, -3 and -4, that encoded the Tfx(1-29) sequence, to the *NheI*/*Apal*-linearized plasmid of pNI-TX1 in a cassette mutagenesis.

[illegible]

D. Construction of the plasmid pNI-TX3

The mutant NI-TX3 is a modified version of NI-TX2, without the extra tetrapeptide ASHA from the (-4) to (-1) positions as in the latter. The plasmid pNI-TX3 was prepared via substitution of the coding sequence for Tfx(1-6) in the NI-TX2 gene with a new coding sequence for TrX (1-6) minus the extra upstream residues. This was accomplished via ligation of the oligonucleotides Tfx(1-6)-1 and Tfx(1-6)-2 into the NheI/PinAI-linearized plasmid pNI-TX2 in a cassette mutagenesis.

Tfx(1-6)-1

Tfx	1	2	3	4	5	6	7	8
fmet	A	V	T	S	N	E	T	G

5-CT AGC TAA GGA GG CTG CAG ATG GCA GTA ACA TCA AAT GAA A
G ATT CCT CC GAC GTC TAC CGT CAT TGT AGT TTA CTT TGG CC
NheI Tfx(1-6)-2 PinAI

E. Construction of the plasmid pNI-TX4

The mutant NI-TX4 is a modified version of NI-TX1, with the (10-29) sequence of the latter substituted by the Tfx(10-29) sequence. The plasmid pNI-TX4 was prepared via substitution of the coding sequence for Tfx(1-6) in NI-TX2 gene with that of TrX (1-6). This was accomplished via ligation of the oligonucleotides TX-1f and TX-8f into the NheI/PinAI-linearized plasmid pNI-TX2 in a cassette mutagenesis.

TX-1f

Tx	1	2	3	4	5	6	7
st	Q	T	I	Q	P	G	T

5'-CT AGC TAA GGA GG CTG CAG ATG CAA ACA ATA CAA CCA GGA A
3'-G ATT CCT CC GAC GTC TAC GTT TGT TAT GTT GGT CCT TGG CC
NheI TX-8f PinAI

F. Construction of the plasmids pNI-TX5 and pTX-28E-29L-162H

The mutant NI-TX5 was identical to NI-TX1, but with the tetrapeptide TYTN aa sequence in the (26-29) region of the latter substituted by the corresponding tetrapeptide SMEL in the (26-29) region of Tfx. The construction of the plasmid pNI-TX5 was through ligation of the heteroduplex oligonucleotides TX-26SMEL-1 and TX-28E/29L-1 (shown below) into NcoI/ApaI-linearized plasmid pNI-TX1 in a the cassette mutagenesis. Subcloning of the plasmid preparation yielded the two target plasmids.

TX-26SMEL-1

22	23	24	25	26	27	28	29	30	31
H	G	G	V	S	M	E	L	G	P

5'-CAT GGT GGT GTG AGC ATG GAG CTC GGG CC
CA CCA CAC TGG ATG CTC GAG C-5'
NcoI T Y ApaI
TX-28E/29L-1

As the two oligonucleotides are heteroduplex, subcloning and hybridization with TX-26SMEL-1 and TX-28E/29L-1 yielded two plasmids, (i) pNI-TX5 with SMEL at the (26-29) region, and (ii) pTX-28E-29L-162H with E and L at positions- 28 and 29.

G. Construction of the plasmid pNI-TX6

The mutant NI-TX6 was identical to NI-TX1, but with two single mutations Y27M and N29L. The construction of the plasmid pNI-TX6 was through ligation of oligonucleotides TX-27M/29L-1 and TX-27M/29L-2 (shown below) into

NcoI/ApaI-linearized plasmid pNI-TX1 in a cassette mutagenesis.

TX-27M/29L-1

```

      22  23  24  25  26  27  28  29  30  31
      H   G   G   V   T   M   T   L   G   P
5' -CAT GGA GGC GTC ACA ATG ACT CTG GGG CC
      CT CCG CAG TGT TAC TGA GAC C
      NcoI                               ApaI

```

TX-27M/29L-2

H. Construction of the plasmid pTX-27M-162H

The mutant TX-27M-162H was identical to NI-TX1, but with an additional mutation Y27M. The construction of the plasmid was through ligation of duplex oligonucleotides TX-27M-1 and TX-27M-2 (shown below) into the NcoI/ApaI-linearized plasmid pNI-TX1 in a cassette mutagenesis.

TX-27M-1

```

      22  23  24  25  26  27  28  29  30  31
      H   G   G   V   T   M   T   N   G   P
5' -CAT GGA GGC GTC ACA ATG ACT AAT GGG CC
      CT CCG CAG TGT TAC TGA TTA C
      NcoI                               ApaI

```

TX-27M-2

I. Construction of the plasmid pNI-TX7

The mutant NI-TX7 is a modified version of NI-TX3, with the tetrapeptide ASAR in the -4 to -1 positions. The construction of the plasmid pNI-TX7 was accomplished via ligation of the oligonucleotides Tf-(-1)R-1 and Tf-(-1)R-2 into the NheI/PinAI-linearized plasmid pNI-TX2 in a cassette mutagenesis.

Tf-(-1)R-1

```

      TfX
      -1  1  2  3  4  5  6  7  8
      A   S   A   R   A   V   T   S   N   E   T   G
5' -CT AGC GCA AGA GCA GTA ACA AGT AAC GAG A
      G CGT TCT CGT CAT TGT TCA TTG CTC TGG CC
      NheI                               PinAI

```

Tf-(-1)R-2

J. Construction of the plasmid pNI-TX8

The mutant NI-TX8 is a modified version of NI-TX3, with the N-terminus of the latter extended upstream (i) with *C. acetobutylicum* xynB (23-31) sequence from (-10) to (-2) positions, and (ii) an Arg residue at (-1) position (Tfx aa numbering). The construction of the plasmid pNI-TX8 was accomplished via ligation of the oligonucleotides CaTf-(-1)R-1 and CaTf-(-1)R-2 into the NheI/PinAI-linearized plasmid pNI-TX2 in a cassette mutagenesis.

	CaTf-(-1)R-1															
	CaX						Tfx									
5	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	
	A	S	A	F	N	T	Q	A	A	P	R	A	V	T	S	N
5	CT	AGC	GCA	TTC	AAC	ACA	CAG	GCC	GCT	CCT	CGA	GCT	GTC	ACC	AGC	AAC
	<u>G</u>		CGT	AAG	TTG	TGT	GTC	CGG	CGA	GGA	GCT	CGA	CAG	TGG	TCG	TTG
	NheI						CaTf-(-1)R2									
10																
	6	7	8													
	E	T	G													
	GAG	A														
15	CTC	<u>TGG</u>	<u>CC</u>													
	PinAI															

K. Construction of the plasmid pNI-TX9

The mutant NI-TX9 is a modified version of NI-TX3, with the N-terminus extended upstream with a tripeptide GRR from the (-3) to (-1) positions (Tfx aa numbering).

PCR primers were constructed to introduce the tripeptide GRR from the (-3) to (-1) positions. The 5' and 3' primers are shown below:

	5' primer, GRR-Tf(1-6)-1															
	TfX															
30				-3	-2	-1	1	2	3	4	5	6	7	8		
			st	G	R	R	A	V	T	S	N	E	T	G		
	5'	ACT	<u>CTG CAG</u>	ATG	GGA	AGA	AGG	GCC	GTA	ACT	TCA	AAT	GAA	ACC	GGT	
		PstI														
35		9	10	11												
		Y	H	D												
		TAT	CAT	GAC												
40																
	3' primer, Uni-PCR-1r															
	5'	GAA	AAG	TGC	CAC	CTG	ACG	TCC	<u>CAA GCT T</u>							
		HindIII														

The plasmid pNI-TX2 was used as a template for PCR. The reaction solution contained the plasmid pNI-TX2 DNA (50 ng, 15 μ L), 5 μ L of 10X buffer (100mM KCl, 100mM ammonium sulfate, 200mM Tris-HCl pH 8.8, 40 mM magnesium sulfate, 1% TritonX-100, 100 μ g/ml BSA), 5 μ L of 5mM dNTPs, 5' primer solution (25 pmol, 2.5 μ L), 3' primer solution (25 pmol, 2.5 μ L) and water (19 μ L).

The reaction was covered with paraffin oil (50 μ L) to prevent evaporation. The reaction mixture was pre-warmed to 94°C without enzyme for 5 min., then the reaction mixture was cooled to 72°C, then the enzyme DNA polymerase (1 μ L, 1U) was added. The reaction was incubated in a temperature cycler for 30 cycles of 94°C for 1 min., 55°C for 2 min. and then 72°C for 2 min. The yield of the PCR product was approximately 1 μ g of a 600 bp fragment. This fragment was purified from an agarose gel.

The resulting PCR product was cut with restriction enzymes PstI and HindIII, and was subsequently ligated into the PstI/HindIII-linearized plasmid pNI-TX3 in a cassette mutagenesis. Transformation in *E. coli* HB101 yielded the plasmid

pNI-TX9.

L. Construction of the plasmid pNI-TX10

- 5 The mutant NI-TX10 was identical to NI-TX1, but with two single mutations H10N and D11N. The construction of the plasmid pNI-TX12 was through ligation of oligonucleotides TX10HD-1 and TX10HD-2 (shown below) into the PinAI/ApaI-linearized plasmid pNI-TX1 in a cassette mutagenesis.

10
 TX10HD-2
 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
 T G F H D G Y F Y S Y W N D G H
 5' CC GGT TTC CAC GAC GGT TAC TTT TAC AGC TAT TGG AAC GAC GGC CAT
 A AAG GTG CTG CCA ATG AAA ATG TCG ATA ACC TTG CTG CCG GTA
 PinAI TX10HD-1
 15
 23 24 25 26 27 28 29 30 31
 G G V T Y T N G P
 GGA GGA GTA ACT TAC ACC AAT GGG CC
 CCT CCT CAT TGA ATG TGG TTA C
 20
 ApaI

25 M. Construction of the plasmid pNI-TX11 and pNI-TX12

- The mutant NI-TX11 and NI-TX12 were identical to NI-TX1 with three and four single mutations respectively. NI-TX11 has three mutations N10H, Y27M and N29L. NI-TX12 has four mutations N10H, N11D, Y27M and N29L.
- 30 The desirable gene has been constructed through PCR with the plasmid pNI-TX6 a template. The 5' and 3' PCR primers TX10HD/N-1 and Uni-PCR-1r, and the PCR protocol for the introduction of the tripeptide GRR have been described in the construction of pNI-TX9.
- The resulting PCR product was cut with restriction enzymes PinAI and HindIII, and was subsequently ligated into the PinAI/HindIII-linearized plasmid pNI-TX1 in a cassette mutagenesis. Transformation in *E. coli* HB101 yielded both
- 35 the plasmids pNI-11 and pNI-12. Their identities were established by nucleotide sequencing.

40
 5' primer TX10HD/N-1
 7 8 9 10 11 12 13 14 15 16 17 18 19 20
 T G Y H D/N G Y F Y S Y W N D
 5' GAA ACC GGT TAC CAC XAC GGT TAC TTT TAC AGC TAT TGG AAC GAT
 Pin AI
 21 22
 G H
 45 GGC C
 X = G + A

50

N. Construction of the plasmid pNI-TX13

- 55 The mutant NI-TX13, is identical to the recombinant wild type TrX, but with the three mutations N10H, Y27M and N29L described in NI-TX11. The generation of this mutant requires the conversion of the residues at positions 1-4 in mutant NI-TX11 to those of TrX. Three residues Ala-1, Ser-2, Gly-4 in mutant NI-TX11 has been converted into Gln, Thr and Gln respectively. The construction of the plasmid pNI-TX13 was through ligation of oligonucleotides TrX1f and TrX8f

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CONSTRUCTION OF THE *BACILLUS CIRCULANS* MUTANT XYLANASES NI-BX

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C. Construction of the plasmid pNI-BX3

The modification in NI-BX3 has been applied in the preparation of NI-TX7. The mutant NI-BX3 is a modified version of NI-BX1, but with its N-terminus extended by one Arg residue at the (-1) position. The construction of the plasmid pNI-BX3 was accomplished via ligation of the oligonucleotides Tf-(-1)R-1 and Tf-(-1)R-2 into the NheI/PinAI-linearized plas-

D. Construction of the plasmid pNI-BX4

The mutant NI-BX4 is identical to NI-BX3, but with an Arg residue replaced by a Lys residue at the (-1) position. The construction of the plasmid pNI-BX4 was accomplished via ligation of the oligonucleotides Tf-(-1)K-1 and Tf-(-1)K-2 into the NheI/PinAI-linearized plasmid pNI-BX1 in a cassette mutagenesis.

15

Tf-(-1)K-1

Tfx

			-1	1	2	3	4	5	6	7	8
	A	S	A	K	A	V	T	S	N	E	T
5'	-CT	AGC	GCA	AAA	GCA	GTA	ACA	AGT	AAC	GAG	A
		<u>G</u>	CGT	TTT	CGT	CAT	TGT	TCA	TTG	CTC	<u>TGG CC</u>
		NheI									PinAI

Tf-(-1)K-2

E. Construction of the plasmids pNI-BX4-K(-1)D and pNI-BX4-K(-1)E

30 The mutant NI-BX4-K(-1)D and NI-BX4-K(-1)E are identical to NI-BX4 or NI-BX3, but with an acidic residue Asp or Glu substituting the basic residue at the (-1) position. The construction of the plasmids was accomplished via ligation of the heteroduplex oligonucleotides Tf-(-1)D-1 and Tf-(-1)E-2 into the NheI/PinAI-linearized plasmid pNI-BX1 in a cassette mutagenesis. Subcloning of the prepared plasmid yielded the target plasmids.

35

Tf-(-1)D-1

Tfx

			-1	1	2	3	4	5	6	7	8
	A	S	A	D	A	V	T	S	N	E	T
5'	-CT	AGC	GCA	GAT	GCA	GTA	ACA	AGT	AAC	GAG	A
		<u>G</u>	CGT	CTT	CGT	CAT	TGT	TCA	TTG	CTC	<u>TGG CC</u>
		NheI		E							PinAI

Tf-(-1)E-2

F. Construction of the plasmid pNI-BX5

55 The modification in NI-BX5 has been applied in the preparation of NI-TX8. The mutant NI-BX5 has (i) a substitution of its (1-29) sequence by the Tfx(1-29) aa sequence, (ii) an extension of an Arg residue at (-1) position (Tfx aa numbering), and (iii) a further extension upstream with an *C. acetobutylicum* xynB (23-31) sequence from (-10) to (-2) positions. The construction of the plasmid pNI-BX5 was accomplished via ligation of the oligonucleotides CaTf-(-1)R-1 and CaTf-(-1)R-2 into the NheI/PinAI-linearized plasmid pNI-BX1 in a cassette mutagenesis.

(B) Purification of modified xylanases

Protein samples were prepared from cells by first making an extract of the cells by grinding 10 g of the cell paste with 25 g of alumina powder. After grinding to smooth mixture, small amounts (5 mL) of ice cold buffer A (10mM sodium acetate, pH 5.5 for BcX mutants) or buffer B (10mM sodium acetate, pH 4.6 for TX mutants) were added and the mixture ground vigorously between additions. The alumina and cell debris were removed by centrifugation of the mixture at 8000 x g for 30 min.

Before column chromatography, the supernatant (25 mL) of modified xylanases were pretreated in one of the following ways:

(1) NI-BX1, NI-BX2, NI-TX1, NI-TX2, NI-TX3, NI-TX4, NI-TX6, NI-TX7, NI-TX10, NI-TX11, NI-TX12, NI-TX13.

Dialysis overnight at 4°C against 3 L buffer A, with dialysis tubing (3500 molecular weight cutoff). A slight precipitate formed in the dialysis bag, which is removed by centrifugation at 8000xg for 15 min.

(2) NI-BX3, NI-BX4, NI-BX5, NI-BX6, NI-BX7

Heating at 60°C for 20 min, then 68°C for 30 min and centrifugation to remove a large amount of precipitate. The supernatant was acidified to pH 4.6, frozen at -20°C overnight thawed and centrifuged to remove more precipitate.

(3) NI-TX8, NI-TX9

Heating at 60°C for 30 min and centrifugation to remove a large amount of precipitate. The supernatant was acidified to pH 4.6, frozen at -20°C overnight, thawed and centrifuged to remove more precipitate.

(4) NI-TX5

Without dialysis or heating, the crude supernatant was directly acidified to pH 4.6, frozen at -20°C overnight, thawed and centrifuged to remove more precipitate.

After the above pretreatment, the cell extract was pumped onto a 50 mL bed volume, S-sepharose fast flow, cation exchange column (Kabi-Pharmacia, Canada), equilibrated in buffer A. The xylanase was eluted with a 300 mL linear gradient of 0 to 0.3M NaCl in buffer A at a flow rate of 3 mL/min. The xylanase elutes at 100 to 150 mL of the gradient. The fractions are checked on SDS-PAGE, and those fractions having most of the xylanase were pooled, and concentrated by ultrafiltration using 3000 dalton molecular weight cutoff membranes (Amicon YM3). The concentrated material (5 mL) was then applied to a 1.5 cm x 85 cm TSK-HW50S gel filtration column, equilibrated in 50 mM ammonium acetate pH 6. The xylanase eluted at a volume of 90 to 100 mL. These fractions were analyzed by SDS-PAGE, and the peaks pooled as pure xylanase. The protein was quantified using the extinction coefficient at 280 nm. Typical purified yield from 10 g of cells was 25 mg of xylanase. All NI-TX and NI-BX xylanases have good solubility in the ammonium acetate buffer without glycerol.

(C) Standard assay for the measurement of enzymatic activity

The quantitative assay determined the number of reducing sugar ends generated from soluble xylan. The substrate for this assay was the fraction of birchwood xylan which dissolved in water from a 5% suspension of birchwood xylan (Sigma Chemical Co.). After removing the insoluble fraction, the supernatant was freeze dried and stored in a dessicator. The measurement of specific activity was performed as follows. Reaction mixtures containing 100 µL of 30 mg/mL xylan previously diluted in assay buffer (50 mM sodium citrate, pH 5.5 or the pH optimum of the tested xylanase), 150 µL assay buffer, 50 µL of enzyme diluted in assay buffer were incubated at 40°C. At various time intervals 50 µL portions were removed and the reaction stopped by diluting in 1 mL of 5mM NaOH. The amount of reducing sugars was determined with the hydroxybenzoic acid hydrazide reagent (HBAH) (Lever, 1972, Analytical Biochem 47:273-279). A unit of enzyme activity was defined as that amount generating 1 µmol reducing sugar in 1 minute at 40°C.

For the comparison between modified and the natural xylanases (Tables 4 and 5), the specific activities of the xylanases was normalized to the specific activity of the natural xylanase.

TABLE 4

Relative activity of NI-TX xylanases	
Xylanase	Relative activity %
natl TrX	100*
TvX(3-190)	102
NI-TX1	98
NI-TX2	92
NI-TX5	95
NI-TX6	99
NI-TX7	90

* The data are normalized to the specific activity of the natural TrX of 770 U/mg.

TABLE 5

Relative activity of NI-BX xylanases	
Xylanase	Relative activity %
BcX	100*
NI-BX1	92
NI-BX3	89
NI-BX6	92

* The data are normalized to the specific activity of BcX of 330 U/mg.

In both NI-TX (Table 4) and NI-BX xylanases (Table 5), the specific enzymatic activities of the modified xylanases at 40°C are not significantly different from the natural xylanases.

EXAMPLE 4

TEMPERATURE/ACTIVITY PROFILES OF MODIFIED TRICHODERMA XYLANASES

This was a measure of the effect of temperature on the enzymatic activity of the xylanase in the hydrolysis of soluble xylan. The procedure was identical to the standard assay with changes in the incubation temperature and time. The enzymes (15 µg/mL) and soluble xylanase in 50 mM sodium citrate buffer of pH 5.5 were mixed and incubated in a circulating water bath at different temperatures. After 30 min, the amount of reducing sugars released from xylan was determined by HBAH and was calculated as a relative activity, with the value at 40°C as 100%.

The effect of temperature on the hydrolysis of xylan is shown in Figure 5. The *E. coli*-expressed TvX(3-190) showed much less activity at 55°C or above than the natural TrX. As reported by Sung et al 1995, similarly low thermophilicity has been shown in the *E. coli*-expressed TrX.

The natural TrX was highly active up to 60°C. The advantage in thermotolerance of this enzyme over that expressed in *E. coli* is probably the result of posttranslational modifications in the *Trichoderma* host.

The mutant NI-TX1, with mutation Q162H, has an activity/temperature profile identical to that of TvX(3-190).

Substitution of the (1-29) region of NI-TX1 with the corresponding sequence of Tfx(1-29) and the N-terminus extension with the tetrapeptide ASHA at the (-4) to (-1) positions generated NI-TX2. If the effective temperature is defined as a temperature allowing 150% relative activity as at 40 °C, this mutant showed a gain of 10°C over TvX(3-190) or NI-

TX1.

A mutant xylanase NI-TX3 with the substitution by Tfx (1-29) but without the N-terminus extension of residues ASHA at the (-4) to the (-1) positions as in NI-TX2, has a temperature/activity profile identical to the latter.

Another mutant NI-TX4 with a substitution of a shorter Tfx(10-29) sequence has a slightly lower thermophilicity than NI-TX2 and NI-TX3.

The chimeric xylanase NI-TX5, with a change of the tetrapeptide from Tfx at the (26-29) region showed a gain of 5 °C over TvX (3-190) or NI-TX1.

The mutant NI-TX6, with double mutations Y27M and N29L retained significant enzymatic activity up to 60°C. It showed the same temperature/activity profile as NI-TX5.

Another mutant NI-TX7 has the same thermophilicity as NI-TX2. NI-TX7 has the same amino acid sequence as NI-TX3 but with the N-terminus extension consisting of a tetrapeptide ASAR in the -4 to -1 positions. Although this mutant and NI-TX2 have shown that extension with these tetrapeptides has no significant effect on the temperature range of the chimeric *Trichoderma* xylanase, other N-terminal extensions have been explored.

NI-TX8 was synthesized and it showed a gain of 13°C over TvX(3-190) or NI-TX1, and about 10°C over natural TrX. It is of the same structure as NI-TX7 with an additional extension upstream of the N-terminus with the *C. acetobutylicum* xynB (23-31) sequence from the (-10) to (-2) positions.

A mutant NI-TX9 was constructed to test whether the *C. acetobutylicum* xynB (23-31) sequence in upstream extension of NI-TX8 can be replaced by a shorter tripeptide sequence GRR. This tripeptide has been identified in the study of NI-BX7 described later. The new mutant NI-TX9 of smaller size showed the same temperature/activity profile as NI-TX8.

This is the first report of enhancement of performance by an upstream extension to the N-terminus of a xylanase.

As for the identification of other residues contributing to the thermophilicity of the Family 11 xylanase, a mutant NI-TX10 was prepared, which was derived from NI-TX1 with two mutations N10H and N11D. The new mutant NI-TX10 showed a gain of 6°C in its effective temperature as compared to its precursor NI-TX1.

Finally two mutant xylanases NI-TX11 and NI-TX12, derivatives of NI-TX1 with the triple mutation (N10H Y27M N29L) and the quadruple mutation (N10H N11D Y27M N29L) respectively, have been constructed. They were used to determine (i) the individual contribution of the mutations N10H and N11D, and (ii) their combined effect with two other advantageous mutations Y27M and N29L identified above. Both mutants NI-TX11 and NI-TX12 have shown identical temperature-activity profile with a gain of 13°C in effective temperature. This result indicated that the N11D mutation has no effect on the thermophilicity of TrX. A conversion of the residues at position (1-4) in NI-TX11 to those of the wild type TrX yielded NI-TX13. The temperature-activity profile of both NI-TX11 and NI-TX13 remained identical.

This gain in the effective temperature for a relative activity of 150% by NI-TX11 or NI-TX13 (+13°C) is greater than the theoretical sum of the gains by individual mutations N10H (+6°C, in NI-TX11) and Y27M/N29L (+4°C, in NI-TX2). This improvement is also greater than any gain via a direct substitution with any natural Tfx sequence which may harbor the triple mutation, as shown in the chimeric mutants NI-TX2 (+10°C), NI-TX3 (+10°C) and NI-TX4 (+9°C). Furthermore, the triple mutation of 3 residues in NI-TX11 or NI-TX13 represents a much smaller modification in TrX, as compared to the substitution with the Tfx sequences of 31-20 residues as in the chimeric enzymes, and this may indirectly minimize other unwanted changes in the general characteristics of the *Trichoderma* xylanase.

EXAMPLE 5

TEMPERATURE/ACTIVITY PROFILES OF MODIFIED BACILLUS XYLANASES

The assay protocol was generally identical to the one for the NI-TX xylanases in Example 4. The effect of temperature on the hydrolysis of soluble xylan in 50 mM sodium citrate buffer of pH 5.5 by the NI-BX xylanases is shown in Figure 6.

The natural BcX was active up to 60°C.

The mutant NI-BX1, with a Tfx(1-31) sequence replacing the BcX(1-22) sequence in its N-terminus, has high activity up to around 82°C. As discussed in the NI-TX section, we can define the effective temperature as a temperature allowing 150% relative activity as in 40°C. In this case, the gain in the thermophilicity of NI-BX1 would be about 22°C. The mutant NI-BX2, without the extra residues ASHA in the (-4) to the (-1) position as in NI-BX1, showed the same temperature/activity profile.

Further modification by the insertion of an Arg residues at the (-1) position in NI-BX3 and NI-BX5 has improved the thermophilicity by 2.5°C over NI-BX1 and NI-BX2.

The same gain has also been observed in NI-BX4 which has another basic residue Lys at the (-1) position. Replacement with acidic residue Glu or Asp causes a loss of around 10°C in maximum temperature (data not shown). Therefore, these results have demonstrated the contribution to the thermotolerance of the extension at the (-1) position with basic amino acids (Arg, Lys), as opposed to the presence of neutral (His) and acidic (Asp, Glu) residues.

The mutant NI-BX6 showed a gain of 2.5°C over NI-BX3 or 5°C over NI-BX1. Thus the thermophilicity can be further improved via extension of the N-terminus through the addition of the *C. acetobutylicum* xynB (23-29) sequence from the (-10) to (-4) positions, Gly at the (-3) position and Arg in both (-2) and (-1) positions.

A mutant NI-BX7 was constructed to test whether the *C. acetobutylicum* xynB (23-31) sequence in upstream extension of NI-BX6 can be replaced by a shorter tripeptide sequence GRR. Although both NI-BX6 and NI-BX7 seemed to have identical effective temperature or top temperature limit, the latter mutant of smaller size showed a wider temperature range for enzymatic activity than the former. As described above in the mutant NI-TX9, the same tripeptide was also successful in elevating the effective temperature of the *Trichoderma* xylanase, without using the CaX sequence.

In summary, the modifications which have successfully increased the thermophilicity of the fungal *Trichoderma* xylanase, are generally applicable in the bacterial *Bacillus circulans* xylanase.

EXAMPLE 6

COMPARISON OF THE THERMOPHILICITY OF THE NI-BX XYLANASES WITH THE CAMPBELL, ET AL PRIOR ART.

The best improved *Bacillus circulans* (BcX) mutant TS19a of the Campbell et al. prior art had modifications to amino acids 3,4,8, and 22 (according to the BcX numbering system). This modified xylanase was compared with the NI-BX xylanases using the same protocols described above, with the results shown in Figure 6. The temperature optima of the NI-BX xylanases are 8-14°C higher than that of TS19a. In addition, at optimum temperature the NI-BX6 and NI-BX7 xylanases of the present invention have 3-fold higher activity than that of Campbell, et al. This set of results demonstrates far superior performance of modified xylanases made by the present invention than with those of Campbell, et al.

EXAMPLE 7

COMPARISON OF THE THERMOPHILICITY OF NI-BX6 AND NI-BX7 WITH THE NATURAL CLOSTRIDIUM AND T. FUSCA XYLANASES

The N-terminal domain of the mutant xylanase NI-BX6 included an extension by a short sequence from the *Clostridium acetobutylicum* xynB (CaX). The N-terminal domain of the mutant xylanase NI-BX7 included an extension by the tripeptide GRR. NI-BX6 and NI-BX7 had a sequence of amino acids and substituted by a short sequence from a thermostable xylanase TfxA of *Thermomonospora fusca*. The thermophilicity of NI-BX6 and NI-BX7 were assessed in comparison with published data on these natural *Thermomonospora* and *Clostridium* xylanases.

The mutants NI-BX6 and NI-BX7, with high activity up to 85°C, are superior to the *Clostridium acetobutylicum* xynB (CaX) with a much lower temperature optimum of 43°C (Zappe et al. 1987, Zappe et al. 1990).

There was no data available on the temperature profile of *T. fusca* xylanase TfxA. The published data on the thermophilicity of the fermentation supernatant of *T. fusca* (Casimir-Schenkel et al. 1992) was available. This supernatant includes six xylanases, of which TfxA is one. The effect of temperature on the activity of NI-BX6 and NI-BX7 was measured according to the protocol described for the fermentation supernatant of *T. fusca* (Casimir-Schenkel et al. 1992). This included the addition of the mutant xylanase to xylan in 50 mM sodium phosphate buffer (pH 7) and incubation at 70, 80 and 90°C for exactly 10 min.

The data shows that the chimeric *Bacillus* xylanase demonstrated greater thermophilicity than the *T. fusca* xylanase supernatant, and hence is very likely greater than TfxA (Table 6).

This example demonstrates the surprising result that insertion of the short sequences in the chimeric modifications can increase the thermotolerance of the xylanase well beyond that of the thermophilic xylanases that are the source of the short sequences.

TABLE 6. Temperature profile of relative enzymatic activity at pH 7.0

Temperature °C	Relative activity (% of maximum)*		
	NI-BX6	NI-BX7 super natan t**	<i>T. fusca</i>
70	100	100	100
80	96	110	58
90	36	20	11

* The enzymatic activity at 70°C was calculated as 100%.

** Published data by Casimir-Schenkel et al. 1992 which is incorporated herein by reference.

In addition to higher temperature for enzymatic activity, some new mutants have also gained greater enzymatic activity, as compared to the wild-type enzyme. The relative activities of some NI-BX xylanases (NI-BX6, NI-BX7) at their respective temperature optima (75°C) were 4-fold as that of the wild type BcX (55°C) (see Figure 6).

In summary, the modifications which have successfully increased the thermophilicity of the fungal *Trichoderma* xylanase, are generally applicable in the bacterial *Bacillus circulans* xylanase.

EXAMPLE 8

PH/ACTIVITY PROFILES OF MODIFIED TRICHODERMA XYLANASES

This was a measure of the effect of different pH on the enzymatic activity of the xylanase in the hydrolysis of soluble xylan. The procedure was identical to the standard assay with changes in the incubation temperature and time. The *Trichoderma* enzymes (15 µg/mL) and soluble xylanase in 50 mM sodium citrate buffers of pH 4-8 were incubated together at 65°C. After 30 min, the amount of reducing sugars released from xylan was determined by HBAH and was expressed as a relative activity, with the value at pH optimum as 100%.

The effect of pH on the enzymatic activity of different NI-TX xylanases was shown in Figure 7.

The natural TrX, NI-TX1, NI-TX5, NI-TX6 showed the same pH/activity profile, with high activity up to pH 5.5 and a significant loss of activity at pH 6.

The substitution with Tfx(1-29) in the mutants NI-TX2, NI-TX3 and NI-TX8 results in full activity up to pH 6.0, and significant activity at pH 7. This represents a gain of 1 pH unit over the natural TrX. The mutant NI-TX9 demonstrated a pH-activity profile identical to NI-TX8, thus indicating the upstream CaX sequence can be replaced by the tripeptide GRR.

The analogue NI-TX10 with the double mutation (N10H N11D) showed a gain of 0.5 pH unit in its upper limit for activity as compared to its precursor NI-TX1. NI-TX11, NI-TX12 and NI-TX13 showed a gain of 0.6 pH unit.

EXAMPLE 9

PH PROFILES OF MODIFIED BACILLUS XYLANASES

The test procedure was identical to the protocol for the modified *Trichoderma* xylanases in Example 8. The *Bacillus circulans* xylanases were incubated with soluble xylan at 65°C in sodium citrate (pH 4-8) and sodium borate buffers (pH 9.5 and 10). As shown in Figure 8, the natural BcX is fully active up to pH 6.0.

The substitution of longer sequence with Tfx(1-33) in the mutants NI-BX1, NI-BX2, NI-BX3, NI-BX5, NI-BX6, and NI-BX7 has extended the activity to above pH 7.0 (Figure 8). This represents a gain of 1.5-2 pH units over BcX.

EXAMPLE 10

COMPARISON OF THE PH/ACTIVITY PROFILE OF THE MODIFIED BACILLUS XYLANASES WITH THAT OF CAMPBELL, ET AL

The pH range of the best xylanase TS19a of the prior art of Campbell et al. was measured and compared with the NI-BX xylanase according to the protocol described in Example 8. The results are shown in Figure 8. The pH optima of the NI-BX xylanases are 1-1.5 unit higher than that of TS19a, while TS19a itself has only a minor improvement (0.5 pH unit) over natural BcX. This result demonstrates the superior performance of the xylanases of the present invention relative to those of Campbell, et al.

EXAMPLE 11

COMPARISON OF THE PH/ACTIVITY PROFILES OF THE MODIFIED *BACILLUS* XYLANASES WITH THE NATURAL XYLANASES OF *CLOSTRIDIUM ACETOBUTYLICUM* AND *T. FUSCA*.

Since the N-terminal domain of the NI-BX xylanases was constituted by short sequences from the *Clostridium acetobutylicum* xynB (CaX) and the xylanase TfxA of *Thermomonospora fusca*, the pH ranges of the modified *Bacillus* xylanases was compared to the published data of these two natural xylanases.

The NI-BX mutant xylanases, with maximal activity up to pH 7 at 65°C (Figure 8), has a higher maximum pH than the *Clostridium acetobutylicum* xynB (CaX) with a pH optimum of 6.0 at 43°C (Zappe et al. 1987, Zappe et al. 1990).

As for the *T. fusca* xylanase TfxA, its pH range has already been disclosed (Wilson et al, PCT/ 1995). For comparative study, the effect of pH on the enzymatic activity of NI-BX xylanases was therefore determined according to the protocol as described for TfxA (Wilson et al, PCT/1995). This included the addition of the xylanase to xylan in 0.05 M sodium glycine buffers of pH 8-10 and incubation at 50°C for 30 min. As shown in Figure 9, the *Bacillus* xylanases NI-BX1, NI-BX2, NI-BX3, NI-BX6, and NI-BX7 all showed pH optima of 9 while the pH optimum of TfxA was 8.

This example shows the surprising result that the xylanases of the present invention have a higher pH range than either of the xylanases used to donate short sequences of amino acids.

EXAMPLE 12

THERMOSTABILITY OF MODIFIED *TRICHODERMA* XYLANASES

This was a measure of the tolerance of xylanase to storage at a set temperature, without any xylan present. The following parameters were generally used in both NI-TX and NI-BX xylanases. The xylanase (150 µg/mL) in assay buffer (50 mM sodium citrate, pH 5.5 or 4.5) was incubated at a set temperature. Aliquots were removed at set intervals. After cooling to 20°C, the residual enzymatic activity of the heated samples was determined via the HBAH assay of Example 3 at 40°C. The enzymatic activity was normalized as a percentage of the activity of the "0 min" aliquot.

Figure 10 shows that chimeric xylanase NI-TX5, NI-TX10, and NI-TX11 have an improved storage stability at 53 °C over the natural TrX. The replacement of the (26-29) region by the SMEL tetrapeptide sequence of Tfx in the case of NI-TX5 has allowed it to retain all of its enzymatic activity after incubation for 60 min.

NI-TX1, TvX(3-190), and Trx expressed in *E. coli* show no improvement in thermostability over the natural TrX.

At a higher incubation temperature of 68°C, the natural TrX, NI-TX1 and NI-TX5 lost all enzymatic activity in less than 10 min (FIG. 11).

At the same temperature, the chimeric xylanase NI-TX2 has retained 40% of enzymatic activity after 10 min.

The chimeric xylanase NI-TX8 has kept 55% of its activity after 60 min, thus representing a tolerance of storage temperature which is about 15°C higher than that by the natural TrX.

In summary, the chimeric modifications in NI-TX2, NI-TX8, and NI-TX9 ie. the replacement of the (1-29) region of the *Trichoderma* xylanase by the Tfx(1-29) sequence, the upstream extension with either the *C. acetobutylicum* xynB (23-31) sequence from the (-10) to (-2) positions and Arg at the (-1) position, or the tripeptide GRR have further increased the thermostability.

EXAMPLE 13

THERMOSTABILITY OF MODIFIED *BACILLUS* XYLANASES

The NI-BX modified *Bacillus* xylanases and BcX were incubated at 70°C and pH 5.5 (Figure 12). The natural xylanase BcX lost all enzymatic activity as expected in less than 10 min. In contrast, the mutant xylanase NI-BX1 retained

most of the activity after 20 to 30 min. This indicated that the thermostability of the *Bacillus circulans* xylanase can be increased via the replacement of the BcX(1-22) sequence by the Tfx(1-31) sequence. Further increase in thermostability among NI-BX3, NI-BX5, NI-BX6, and NI-BX7 was achieved by the extension of the N-terminus through either the addition of the *C. acetobutylicum* xynB (23-29) sequence from the (-10) to (-4) positions, Gly at the (-3) position and Arg in both (-2) and (-1) positions or the tripeptide GRR. For NI-BX6 and NI-BX7, the gain in thermostability is about 15°C relative to the natural enzyme BcX. Thus the modifications that successfully increase the thermostability of the fungal *Trichoderma* xylanase are generally applicable in the *Bacillus circulans* xylanase.

EXAMPLE 14

EVALUATION OF PERFORMANCE OF MODIFIED TRICHODERMA XYLANASES IN TREATMENT OF PULP

The assay described above in the Examples 3 through 13 involves hydrolysis of soluble xylan and this procedure has been successful in the identification of thermophilic modified xylanases in the NI-TX and NI-BX series. However, in the pretreatment of pulp in the bleaching process, xylanase would interact with the insoluble xylan in pulp. It is therefore important to confirm that the performance improvements identified using the soluble xylan substrate would be observed in the treatment of pulp. Therefore, the performance of the modified xylanases was evaluated in the treatment of brown-stock pulp prior to bleaching.

Samples of natural and modified enzymes were sent to logen Corporation of Ottawa, Canada for testing on pulp. logen manufactures and supplies xylanase enzymes to the pulp industry and has developed tests to assess the performance of xylanase enzymes in treating pulp. The test involves adding the enzymes to pulp for a specified period and then measuring the effect of the enzyme on the subsequent bleachability of the pulp. The test were carried out using a commercial softwood Kraft pulp of Kappa number 25.6.

The results of the logen pulp testing of *Trichoderma* xylanases is shown in Figure 13. At pH 6.0, the optimum pH for the natural TrX in treating pulp, good performance was achieved up to 52°C. The natural TrX was not active on pulp at pH 6.5.

The *E. coli*-expressed NI-TX1 with a single point mutation Q162H was ineffective on pulp at temperatures higher than 40°C. By contrast, the mutants NI-TX5, NI-TX2 and NI-TX8 could function up to 60, 61, and 65°C respectively at pH 6.5.

Although the absolute temperatures tolerated by the enzymes are lower on pulp than in the hydrolysis of soluble xylan, the improvements by the modified xylanases in temperature (+8 to 13°C) and pH (+0.5 unit) in the treatment of pulp were consistent with the gains in their thermophilicity and alkalophilicity in xylan hydrolysis described in Examples 4 and 8.

The temperature and pH ranges tolerated by the enzymes in pulp treatment is encouraging, based on these preliminary tests. Much more extensive testing of temperature and pH range using different pulps and treatment techniques would likely increase the range of conditions in which the modified xylanases are effective on pulp.

EXAMPLE 15

EVALUATION OF PERFORMANCE OF MODIFIED BACILLUS XYLANASES IN TREATMENT OF PULP

The *B. circulans* xylanases were also tested in the treatment of pulp by logen Corporation (Figure 14).

At pH 8.0, the natural BcX showed poor performance at temperatures higher than 40°C. When the pH was lowered to 7, BcX was only effective up to 50°C.

By contrast, the mutant NI-BX1 was active up to 75°C at pH 8.0.

Although the absolute temperature tolerated by the NI-BX1 enzyme is lower on pulp than in the hydrolysis of soluble xylan, the improvements by the modified xylanase in temperature (+28°C) and pH (+1 unit) in the treatment of pulp were consistent with the gains in their temperature tolerance and pH range in xylan hydrolysis described in Examples 5 and 9.

The temperature and pH ranges tolerated by the enzymes in pulp treatment is encouraging, based on these preliminary tests. Much more extensive testing of temperature and pH range using different pulps and treatment techniques would likely increase the range of conditions in which the modified xylanases are effective on pulp.

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10 Lüthi, E., Jasmat, N. B., and Bergquist, P. L. (1990) *Appl. Environ. Microbiol.* 56:2677-2683.

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SEQUENCE LISTING

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- (B) STREET: 1200 Montreal Road
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- (D) STATE: Ontario
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- (F) POSTAL CODE (ZIP): K1A 0R6

(ii) TITLE OF INVENTION: MODIFICATION OF XYLANASE TO IMPROVE
THERMOPHILICITY, ALKOPHILICITY AND THERMOSTABILITY

(iii) NUMBER OF SEQUENCES: 54

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 184 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Aspergillus niger*, var. *awamori*

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Moat Dr, J
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Hessing, J.G.M.
Hondel, C.A.M.J
- (C) JOURNAL: Xylan and Xylanase
- (F) PAGES: 349-360
- (G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ser Ala Gly Ile Asn Tyr Val Gln Asn Tyr Asn Gly Asn Leu Gly Asp
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 20 25 30
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 35 40 45
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 Ser Tyr Leu Ala Val Tyr Gly Trp Val Asn Tyr Pro Gly Ala Glu Tyr
 65 70 75 80
 Tyr Ile Val Glu Asp Tyr Gly Asp Tyr Asn Pro Cys Ser Ser Ala Thr
 85 90 95
 Ser Leu Gly Thr Val Tyr Ser Asp Gly Ser Thr Tyr Gln Val Cys Thr
 100 105 110
 Asp Thr Arg Ile Asn Glu Pro Ser Ile Thr Gly Thr Ser Thr Phe Thr
 115 120 125
 Gln Tyr Phe Ser Val Arg Glu Ser Thr Arg Thr Ser Gly Thr Val Thr
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 Val Ala Asn His Phe Asn Phe Trp Ala Gln His Gly Phe Gly Asn Ser
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 185 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Aspergillus tubigenis*

(x) PUBLICATION INFORMATION:

(A) AUTHORS: de Graaff, L.H.
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van Ooijan, A.J.J.
Visser, J

(C) JOURNAL: Xylan and Xylanase

(F) PAGES: 235-246

(G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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85     90     95
Thr Ser Leu Gly Thr Val Tyr Ser Asp Gly Ser Thr Tyr Gln Val Cys
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130    135    140
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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 185 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacillus circulans

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Yang, R.C.A.

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(C) JOURNAL: Nucleic Acid Research

(D) VOLUME: 16

(F) PAGES: 7187

(G) DATE: 1988

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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35 40 45
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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 201 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Bacillus pumilus*

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Fukusaki, E
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Okada, H

(C) JOURNAL: FEBS Letters

(D) VOLUME: 171

(F) PAGES: 197-201

(G) DATE: 1984

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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- (A) LENGTH: 185 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Bacillus subtilis*

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Parce, M.G.
 Bourbonnais, R
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 (C) JOURNAL: Arch. Microbiol.
 (D) VOLUME: 144
 (F) PAGES: 201-206
 (G) DATE: 1986

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Ile Val
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(2) INFORMATION FOR SEQ ID NO:7:

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 206 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 25 (ii) MOLECULE TYPE: protein
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 30 (v) FRAGMENT TYPE: internal
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Clostridium stercorarium
 (B) STRAIN: Xyn A
 35 (x) PUBLICATION INFORMATION:
 (A) AUTHORS: Sakka, K
 Kojima, Y
 Kondo, T
 40 Karita, S
 Ohmiya, K
 Shimada, K
 (C) JOURNAL: Biosci, Biotech, Biochem
 (D) VOLUME: 57
 (F) PAGES: 273-277
 45 (G) DATE: 1993

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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 50 55 60
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 65 70 75 80
 10 Tyr Leu Cys Val Tyr Gly Trp Thr Arg Asn Phe Leu Val Glu Tyr Tyr
 85 90 95
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 15 Gly Thr Ile Thr Gln Trp Met Ala Gly Thr Tyr Glu Ile Tyr Glu Thr
 115 120 125
 Thr Arg Val Asn Gln Pro Ser Ile Asp Gly Thr Ala Thr Phe Gln Gln
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 20 Tyr Trp Ser Val Arg Thr Ser Lys Arg Thr Ser Gly Thr Ile Ser Val
 145 150 155 160
 Thr Glu His Phe Lys Gln Trp Glu Arg Met Gly Met Arg Met Gly Lys
 165 170 175
 25 Met Tyr Glu Val Ala Leu Thr Val Glu Gly Tyr Gln Ser Ser Gly Tyr
 180 185 190
 Ala Asn Val Tyr Lys Asn Glu Ile Arg Ile Gly Ala Asn Pro
 195 200 205
 30

(2) INFORMATION FOR SEQ ID NO:8:

- 35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 211 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 40 (ii) MOLECULE TYPE: protein
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 (v) FRAGMENT TYPE: internal
 45 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Ruminococcus flavefaciens*
 (x) PUBLICATION INFORMATION:
 (A) AUTHORS: Zhang, J
 Flint, H. J.
 50 (C) JOURNAL: EMBL database accession number Z11127
 (G) DATE: 1992
 55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

5 Ser Ala Ala Asp Gln Gln Thr Arg Gly Asn Val Gly Gly Tyr Asp Tyr
 1 5 10 15
 Glu Met Trp Asn Gln Asn Gly Gln Gly Gln Ala Ser Met Asn Pro Gly
 20 25 30
 10 Ala Gly Ser Phe Thr Cys Ser Trp Ser Asn Ile Glu Asn Phe Leu Ala
 35 40 45
 Arg Met Gly Lys Asn Tyr Asp Ser Gln Lys Lys Asn Tyr Lys Ala Phe
 50 55 60
 15 Gly Asn Ile Val Leu Thr Tyr Asp Val Glu Tyr Thr Pro Arg Gly Asn
 65 70 75 80
 Ser Tyr Met Cys Val Tyr Gly Trp Thr Arg Asn Pro Leu Met Glu Tyr
 85 90 95
 20 Tyr Ile Val Glu Gly Trp Gly Asp Trp Arg Pro Pro Gly Asn Asp Gly
 100 105 110
 Glu Val Lys Gly Thr Val Ser Ala Asn Gly Asn Thr Tyr Asp Ile Arg
 115 120 125
 25 Lys Thr Met Arg Tyr Asn Gln Pro Ser Leu Asp Gly Thr Ala Thr Phe
 130 135 140
 Pro Gln Tyr Trp Ser Val Arg Gln Thr Ser Gly Ser Ala Asn Asn Gln
 145 150 155 160
 30 Thr Asn Tyr Met Lys Gly Thr Ile Asp Val Ser Lys His Phe Asp Ala
 165 170 175
 Trp Ser Ala Ala Gly Leu Asp Met Ser Gly Thr Leu Tyr Glu Val Ser
 180 185 190
 35 Leu Asn Ile Glu Gly Tyr Arg Ser Asn Gly Ser Ala Asn Val Lys Ser
 195 200 205
 Val Ser Val
 210

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 197 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Schizophyllum commune
(B) STRAIN: Xylanase A

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Oku, T
Yaguchi, M
Parse, M
Jurasek, L
(C) JOURNAL: Canadian Fed. Biol. Soc. annual meeting
(F) PAGES: Abstract #676
(G) DATE: 1988

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ser Gly Thr Pro Ser Ser Thr Gly Thr Asp Gly Gly Tyr Tyr Tyr Ser
1 5 10 15
Trp Trp Thr Asp Gly Ala Gly Asp Ala Thr Tyr Gln Asn Asn Gly Gly
20 25 30
Gly Ser Tyr Thr Leu Thr Trp Ser Gly Asn Asn Gly Asn Leu Val Gly
35 40 45
Gly Lys Gly Trp Asn Pro Gly Ala Ala Ser Arg Ser Ile Ser Tyr Ser
50 55 60
Gly Thr Tyr Gln Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp
65 70 75 80
Thr Arg Ser Ser Leu Ile Glu Tyr Tyr Ile Val Glu Ser Tyr Gly Ser
85 90 95
Tyr Asp Pro Ser Ser Ala Ala Ser His Lys Gly Ser Val Thr Cys Asn
100 105 110
Gly Ala Thr Tyr Asp Ile Leu Ser Thr Trp Arg Tyr Asn Ala Pro Ser
115 120 125
Ile Asp Gly Thr Gln Thr Phe Glu Gln Phe Trp Ser Val Arg Asn Pro
130 135 140
Lys Lys Ala Pro Gly Gly Ser Ile Ser Gly Thr Val Asp Val Gln Cys
145 150 155 160
His Phe Asp Ala Trp Lys Gly Leu Gly Met Asn Leu Gly Ser Glu His
165 170 175
Asn Tyr Gln Ile Val Ala Thr Glu Gly Tyr Gln Ser Ser Gly Thr Ala
180 185 190
Thr Ile Thr Val Thr
195

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 191 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Streptomyces lividans

(B) STRAIN: Xln B

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Shareck, F

Roy, C

Yaguchi, M

Morosoli, R

Kluepfel, D

(C) JOURNAL: Gene

(D) VOLUME: 107

(F) PAGES: 75-82

(G) DATE: 1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Asp Thr Val Val Thr Thr Asn Gln Glu Gly Thr Asn Asn Gly Tyr Tyr
 1 5 10 15

Tyr Ser Phe Trp Thr Asp Ser Gln Gly Thr Val Ser Met Asn Met Gly
 20 25 30

Ser Gly Gly Gln Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val
 35 40 45

Ala Gly Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Gln Tyr Ser
 50 55 60

Gly Ser Phe Asn Pro Ser Gly Asn Ala Tyr Leu Ala Leu Tyr Gly Trp
 65 70 75 80

Thr Ser Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Thr
 85 90 95

Tyr Arg Pro Thr Gly Glu Tyr Lys Gly Thr Val Thr Ser Asp Gly Gly
 100 105 110

Thr Tyr Asp Ile Tyr Lys Thr Thr Arg Val Asn Lys Pro Ser Val Glu
 115 120 125

Gly Thr Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg
 130 135 140

Thr Gly Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
 145 150 155 160

Ala Gly Met Pro Leu Gly Asn Phe Ser Tyr Tyr Met Ile Asn Ala Thr
 165 170 175

Glu Gly Tyr Gln Ser Ser Gly Thr Ser Ser Ile Asn Val Gly Gly

180

185

190

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 191 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Streptomyces lividans

(B) STRAIN: Xln C

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Shareck, F

Roy, C

Yaguchi, M

Morosoli, R

Kluepfel, D

(C) JOURNAL: Gene

(D) VOLUME: 107

(F) PAGES: 75-82

(G) DATE: 1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Thr Thr Ile Thr Thr Asn Gln Thr Gly Thr Asp Gly Met Tyr Tyr
1 5 10 15

Ser Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly
20 25 30

Gly Gly Ser Tyr Ser Thr Gln Trp Thr Asn Cys Gly Asn Phe Val Ala
35 40 45

Gly Lys Gly Trp Ser Thr Gly Asp Gly Asn Val Arg Tyr Asn Gly Tyr
50 55 60

Phe Asn Pro Val Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
85 90 95

Pro Thr Gly Thr Tyr Lys Gly Thr Val Ser Ser Asp Gly Gly Thr Tyr
100 105 110

Asp Ile Tyr Gln Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Gly Thr
115 120 125

Lys Thr Phe Gln Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
 130 135 140
 5 Gly Ser Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
 145 150 155 160
 Ala Gly Met Asn Met Gly Gln Phe Arg Tyr Tyr Met Ile Asn Ala Thr
 165 170 175
 10 Glu Gly Tyr Gln Ser Ser Gly Ser Ser Asn Ile Thr Val Ser Gly
 180 185 190

(2) INFORMATION FOR SEQ ID NO:12:

- 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 189 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 20 (ii) MOLECULE TYPE: protein
 (iii) HYPOTHETICAL: NO
 25 (iv) ANTI-SENSE: NO
 (v) FRAGMENT TYPE: internal
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Streptomyces sp. 36a
 30 (x) PUBLICATION INFORMATION:
 (A) AUTHORS: Nagashima, M
 Okumoto, Y
 Okanishi, M
 (C) JOURNAL: Trends in Actinomycetologia
 35 (F) PAGES: 91-96
 (G) DATE: 1989

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Thr Thr Ile Thr Asn Glu Thr Gly Tyr Asp Gly Met Tyr Tyr Ser
 1 5 10 15
 40 Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly Gly
 20 25 30
 Gly Ser Tyr Ser Thr Arg Trp Thr Asn Cys Gly Asn Phe Val Ala Gly
 35 40 45
 45 Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Arg Tyr Thr Gly Trp
 50 55 60
 Phe Asn Pro Ser Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
 65 70 75 80
 50 Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
 85 90 95

Pro Thr Gly Glu Thr Arg Gly Thr Val His Ser Asp Gly Gly Thr Tyr
100 105 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Ala Pro
115 120 125

Ala Ala Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
130 135 140

Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg Ala Gly
145 150 155 160

Met Asn Met Gly Asn Phe Arg Tyr Tyr Met Ile Asn Ala Thr Glu Gly
165 170 175

Tyr Gln Ser Ser Gly Ser Ser Thr Ile Thr Val Ser Gly
180 185

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 189 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Thermomonospora fusca
- (B) STRAIN: Tfx A

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Irwin, D
Jung, E. D.
Wilson, D. B.
- (C) JOURNAL: Appl. Environ. Microbiol.
- (D) VOLUME: 60
- (F) PAGES: 763-770
- (G) DATE: 1994

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ala Val Thr Ser Asn Glu Thr Gly Tyr His Asp Gly Tyr Phe Tyr Ser
1 5 10 15

Phe Trp Thr Asp Ala Pro Gly Thr Val Ser Met Glu Leu Gly Pro Gly
20 25 30

Gly Asn Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val Ala Gly
35 40 45

Lys Gly Trp Ala Thr Gly Gly Arg Arg Thr Val Thr Tyr Ser Ala Ser
50 55 60

Phe Asn Pro Ser Gly Asn Ala Tyr Leu Thr Leu Tyr Gly Trp Thr Arg
 65 70 75 80
 5 Asn Pro Leu Val Glu Tyr Tyr Ile Val Glu Ser Trp Gly Thr Tyr Arg
 85 90 95
 Pro Thr Gly Thr Tyr Met Gly Thr Val Thr Thr Asp Gly Gly Thr Tyr
 100 105 110
 10 Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Ile Glu Gly Thr
 115 120 125
 Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg Thr Ser
 130 135 140
 15 Gly Thr Ile Thr Ala Gly Asn His Phe Asp Ala Trp Ala Arg His Gly
 145 150 155 160
 Met His Leu Gly Thr His Asp Tyr Met Ile Met Ala Thr Glu Gly Tyr
 165 170 175
 20 Gln Ser Ser Gly Ser Ser Asn Val Thr Leu Gly Thr Ser
 180 185

(2) INFORMATION FOR SEQ ID NO:14:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 190 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 30 (ii) MOLECULE TYPE: protein
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 35 (v) FRAGMENT TYPE: internal
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Trichoderma harzianum*
 40 (x) PUBLICATION INFORMATION:
 (A) AUTHORS: Yaguchi, M
 Roy, C
 Watson, D. C.
 Rollin, F
 Tan, L. U. L.
 Senior, D. J.
 45 Saddler, J. N.
 (C) JOURNAL: Xylan and Xylanase
 (F) PAGES: 435-438
 (G) DATE: 1992
 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
 Gln Thr Ile Gly Pro Gly Thr Gly Tyr Ser Asn Gly Tyr Tyr Tyr Ser
 1 5 10 15

55

Tyr Trp Asn Asp Gly His Ala Gly Val Thr Tyr Thr Asn Gly Gly Gly
 20 25 30
 Gly Ser Phe Thr Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
 35 40 45
 Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
 50 55 60
 Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Ile Tyr Gly Trp Ser
 65 70 75 80
 Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
 85 90 95
 Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
 100 105 110
 Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
 115 120 125
 Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
 130 135 140
 Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
 145 150 155 160
 Ser His Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
 165 170 175
 Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
 180 185 190

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 178 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Trichoderma reesei*
- (B) STRAIN: Xyn I

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Torronene, A
Mach, R. L.
Messner, R.
Gonzalez, R.
Kalkkinen, N

Harkki, A
Kubicek, C. P.

(C) JOURNAL: BioTechnology
(D) VOLUME: 10
(F) PAGES: 1461-1465
(G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ala Ser Ile Asn Tyr Asp Gln Asn Tyr Gln Thr Gly Gly Gln Val Ser
1 5 10 15
Tyr Ser Pro Ser Asn Thr Gly Phe Ser Val Asn Trp Asn Thr Gln Asp
20 25 30
Asp Phe Val Val Gly Val Gly Trp Thr Thr Gly Ser Ser Ala Pro Ile
35 40 45
Asn Phe Gly Gly Ser Phe Ser Val Asn Ser Gly Thr Gly Leu Leu Ser
50 55 60
Val Tyr Gly Trp Ser Thr Asn Pro Leu Val Glu Tyr Tyr Ile Met Glu
65 70 75 80
Asp Asn His Asn Tyr Pro Ala Gln Gly Thr Val Lys Gly Thr Val Thr
85 90 95
Ser Asp Gly Ala Thr Tyr Thr Ile Trp Glu Asn Thr Arg Val Asn Glu
100 105 110
Pro Ser Ile Gln Gly Thr Ala Thr Phe Asn Gln Tyr Ile Ser Val Arg
115 120 125
Asn Ser Pro Arg Thr Ser Gly Thr Val Thr Val Gln Asn His Phe Asn
130 135 140
Trp Ala Ser Leu Gly Leu His Leu Gly Gln Met Met Asn Tyr Gln Val
145 150 155 160
Val Ala Val Glu Gly Trp Gly Gly Ser Gly Ser Ala Ser Gln Ser Val
165 170 175
Ser Asn

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 190 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Trichoderma reesei*
 (B) STRAIN: Xyn II

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Torronene, A
 Mach, R. L.
 Messner, R
 Gonzalez, R
 Kalkkinen, N
 Harkki, A
 Kubicek, C. P.
 (C) JOURNAL: *Biotechnology*
 (D) VOLUME: 10
 (F) PAGES: 1461-1465
 (G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gln Thr Ile Gln Pro Gly Thr Gly Tyr Asn Asn Gly Tyr Phe Tyr Ser
 1 5 10 15
 Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
 20 25 30
 Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
 35 40 45
 Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
 50 55 60
 Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
 65 70 75 80
 Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
 85 90 95
 Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
 100 105 110
 Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
 115 120 125
 Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
 130 135 140
 Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
 145 150 155 160
 Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
 165 170 175
 Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
 180 185 190

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 190 amino acids

(B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Trichoderma viride*

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Yaguchi, M
 Roy, C
 Ujie, M
 Watson, D. C.
 Wakarchuk, W.

(C) JOURNAL: Xylan and Xylanase

(F) PAGES: 149-154

(G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```

Gln Thr Ile Gln Pro Gly Thr Gly Phe Asn Asn Gly Tyr Phe Tyr Ser
1          5          10          15
Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
20          25          30
Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35          40          45
Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
50          55          60
Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65          70          75          80
Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85          90          95
Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100         105         110
Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115         120         125
Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Thr His
130         135         140
Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145         150         155         160
Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
165         170         175

```

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser

180

185

190

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 573 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pTvX(3-190)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```

CTAGCATAGG ACCAGGAACC GGTTCACAAC ACGGTTACTT TTACAGCTAT TGGAACGATG   60
GCCATGGTGG TGTTACCTAT ACAAACGGGC CCGGAGGCCA ATTTAGCGTC AATTGGTCTA  120
ACTCCGGAAC CTTCGTAGGT GGAAAAGGTT GGCAACCCGG GACCAAAAAT AAGGTGATCA  180
ACTTCTCTGG ATCTTATAAT CCGAATGGGA ATTCATACTT AAGCGTCTAT GGCTGGTCTA  240
GAAACCCACT GATTGAATAT TACATTGTCTG AAAATTTCGG TACCTACAAT CCGAGTACCG  300
GCGCCACAAA ATTAGGCGAA GTCAGTAGTG ATGGATCCGT ATATGATATC TACCGTACCC  360
AACGCGTTAA TCAGCCATCG ATCATTGGAA CCGCCACCTT TTATCAGTAC TGGAGTGTTA  420
GACGTACGCA TCGGAGCTCC GGTTCGGTTA ATACTGCGAA TCACTTTAAT GCATGGGCAC  480
AGCAAGGGTT AACCCTAGGT ACAATGGATT ATCAAATCGT AGCGGTGGAA GGCTACTTCT  540
CGAGTGTTTC CGCTAGTATT ACAGTGAGCT AAA                               573

```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 579 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: pXYbc

(viii) POSITION IN GENOME:
(C) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```

10 GCTAGCACAG ATTACTGGCA AACTGGACA GACGGTGGCG GTATCGTTAA TGCCGTGAAC 60
   GGCTCCGGAG GCAACTACAG CGTGAATTGG TCTAATACTG GGAAC TTCGT AGTCGGAAAA 120
   GGT TGGACGA CAGGATCCCC GTTCCGTACG ATCAACTACA ACGCTGGCGT TTGGGCCCCG 180
15 AATGGTAACG GTTACCTGAC ACTGTATGGC TGGACGCGTT CGCCACTGAT TGAATATTAC 240
   GTTGTGCGACT CTTGGGGAAC GTACCGTCCG ACTGGAACCT ACAAAGGCAC AGTCAAAAGC 300
   GATGGTGGTA CCTATGACAT CTACACCACC ACAAGATACA ACGCACCTTC CATCGATGGC 360
20 GATCGGACCA CCTTTACTCA GTATTGGAGT GTTAGACAAT CTAAGCGGCC GACTGGTTTCG 420
   AACGCCACCA TTACGTTTAC CAATCACGTG AATGCATGGA AATCCCACGG TATGAACCTA 480
   GGT TCTAATT GGGCTTATCA AGTAATGGCG ACCGAAGGCT ACCAGAGCTC TGTTCTTCC 540
25 AACGTTACAG TGTGGTAAAG ATCTTGAAGC TTGGGACGT 579

```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-162H-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```

45 TGGGCACAGC ACGGGTTAAC C 21

```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-162H-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTAGGGTTAA CCCGTGCTGT GCCCATGCA

29

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTAGCCACGC GGCCGTAAC TCAAATGAAA CCGGTTATCA TGACGGCTAT TTCTACAGCT
TCTGG

60
65

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

ACCGATGCAC CGGGAACTGT GTCCATGGAG CTCGGGCC

38

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Tfx-3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCATGATAAC CGGTTTCATT TGAAGTTACG GCCGCGTGG

39

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 56 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Tfx-4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CGAGCTCCAT GGACACAGTT CCCGGTGCAT CGGTCCAGAA GCTGTAGAAA TAGCCG

56

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx(1-6)-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CTAGCTAAGG AGGCTGCAGA TGGCAGTAAC ATCAAATGAA A

41

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx(1-6)-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCGGTTTCAT TTGATGTTAC TGCCATCTGC AGCCTCCTTA G

41

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-1f

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTAGCTAAGG AGGCTGCAGA TGCAAACAAT ACAACCAGGA A

41

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-8f

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCGGTTCCTG GTTGTATTGT TTGCATCTGC AGCCTCCTTA G

41

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-26SMEL-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CATGGTGGTG TGAGCATGGA GCTCGGGCC

29

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-28E/29L-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CGAGCTCGTA GGTACACCA C

21

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

- (B) CLONE: TX-27M/29L-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CATGGAGGCG TCACAATGAC TCTGGGGCC

29

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

- (B) CLONE: TX-27M/29L-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCAGAGTCAT TGTGACGCCT C

21

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-27M-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:
CATGGAGGCG TCACAATGAC TAATGGGCC

29

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-27M-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:
CATTAGTCAT TGTGACGCCT C

21

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)R-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:
CTAGCGCAAG AGCAGTAACA AGTAACGAGA

30

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)R-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCGGTCTCGT TACTTGTTAC TGCTCTTGCG

30

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: CaTf-(-1)R-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CTAGCGCATT CAACACACAG GCCGCTCCTC GAGCTGTCAC CAGCAAC

47

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: CaTf-(-1)R2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCGGTCTCGT TGCTGGTGAC AGCTCGAGGA GCGGCCTGTG TGTGAATGC G

51

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

- (B) CLONE: GRR-Tf(1-6)-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

ACTCTGCAGA TGGGAAGAAG GGCCGTAAC TCAAATGAAA CCGGTTATCA TGAC

54

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Uni-PCR-1r

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GAAAAGTGCC ACCTGACGTC CCAAGCTT

28

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 73 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX10HD-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CCGGTTTCCA CGACGGTTAC TTTTACAGCT ATTGGAACGA CGGCCATGGA GGAGTAACTT 60
ACACCAATGG GCC 73

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX10HD-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CATTGGTGTA AGTTACTCCT CCATGGCCGT CGTTCCAATA GCTGTAAAAG TAACCGTCGT 60
GGAAA 65

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX10HD/N-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GAAACCGGTT ACCACRACGG TTACTTTTAC AGCTATTGGA ACGATGGCC 49

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TrX1f

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CTAGCTAAGG AGGCTGCAGA TGCAAACAAT ACAACCAGGA A

41

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TrX8f

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CCGGTTCCTG GTTGTATTGT TTGCATCTGC AGCCTCCTTA G

41

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx-2b

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

ACCGATGCCC CGGGAAGTGT GAGTATGGAG CTCGGCC

37

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: Tfx-4b

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CCGGGGCCGA GCTCCATACT CACAGTCCC GGGGCATCGG TCCAGAAGCT GTAGAAATAG 60

CCG

63

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: Tf-(-1)K-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CTAGCGCAAA AGCAGTAACA AGTAACGAGA

30

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

5 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)K-2

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CCGGTCTCGT TACTTGTTAC TGCTTTTGCG

30

15 (2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

25 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)D-1

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

CTAGCGCAGA TGCAGTAACA AGTAACGAGA

30

35 (2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

45 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)E-2

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

CCGGTCTCGT TACTTGTTAC TGCTTCTGCG

30

55

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 61 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Catf-PCR-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CCCGCTAGCG CATTCAACAC ACAAGCARGT SSAAGGGCCG TAACTTCAAA TGAAACCGGT 60
 T 61

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Xy-14a

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

ATTCGGGGCC CAAACGCCAG CGTTGTAGTT GATCGTACG 39

Claims

1. A Family 11 xylanase enzyme selected from the group consisting of the Family 11 xylanases of *Trichoderma*, *Aspergillus*, *Streptomyces*, and *Bacillus* having the amino acid tyrosine or phenylalanine in position 14 as determined by the amino acid numbering of *Trichoderma reesei* xylanase II or the corresponding position in the other xylanases when the amino acids are aligned and having at least 8 amino acid residues in a N-terminus upstream from position 10, said xylanase enzyme having been modified to exhibit enhanced thermophilicity, alkalophilicity, or thermostability with respect to a naturally-occurring enzyme by replacing an amino acid at position 10 with a different amino acid.
2. A modified xylanase according to claim 1 wherein the Family 11 xylanase is selected from the group consisting of *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma harzianum* Xylanase, *Trichoderma*

viride Xylanase, *Streptomyces lividans* xylanase B, and *Streptomyces lividans* xylanase C.

3. A modified xylanase according to claim 1 wherein amino acid 10 is substituted with another acid, and amino acids 27 and 29 are substituted with amino acids selected from the group consisting of valine, methionine, isoleucine, and leucine.
4. A modified xylanase according to claim 3 wherein amino acids 10, 27, and 29 are substituted with histidine, methionine, and leucine, respectively.
5. A Family 11 xylanase enzyme selected from the group consisting of the Family 11 xylanases of *Trichoderma*, *Aspergillus*, *Streptomyces*, and *Bacillus* having the amino acid tyrosine or phenylalanine in position 14 as determined by the amino acid numbering of *Trichoderma reesei* xylanase II or the corresponding position in the other xylanases when the amino acids are aligned, said xylanase enzyme having been modified to exhibit enhanced thermophilicity, alkalophilicity, or thermostability by substituting a sequence of amino acids in a N-terminal region of said xylanase enzyme with that of a corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A to form a chimeric xylanase, and an upstream extension of the N-terminus of the chimeric xylanase with the addition of from 0 to 10 amino acids.
6. A modified xylanase according to claim 5 wherein the Family 11 xylanase is selected from the group consisting of *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma harzianum* xylanase, *Trichoderma viride* xylanase, *Bacillus circulans* xylanase A, *Bacillus subtilis* xylanase A, *Aspergillus niger* xylanase A, *Aspergillus kawachii* xylanase C, *Aspergillus tubigensis* xylanase A, *Streptomyces lividans* xylanase B, and *Streptomyces lividans* xylanase C.
7. A modified xylanase according to claim 1 wherein the sequence of amino acids comprising amino acids 10-29 is substituted by the corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A.
8. A modified xylanase according to claim 5 wherein the sequence of amino acids comprising amino acids 1-29 is substituted by the corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A.
9. A modified xylanase according to claim 5 wherein said xylanase is selected from the group consisting of NI-TX2, NI-TX3, NI-TX4, NI-TX5, NI-TX7, NI-TX8, NI-TX9, NI-BX1, NI-BX2, NI-BX3, NI-BX4, NI-BX5, NI-BX6 and NI-BX7.
10. A modified xylanase according to claim 8 wherein the upstream extension of the N-terminus consists of the addition of one or two basic amino acids in the -2 and -1 positions and the (23-31) region of *Clostridium acetobutylicum* xylanase xynB in the -10 to -3 positions.
11. A modified xylanase according to claim 5 wherein the upstream extension of the N-terminus comprises the addition of any of the tetrapeptides identified by the sequences Alanine-Serine-Alanine-Arginine or Alanine-Serine-Alanine-Lysine.
12. A modified xylanase according to claim 8 wherein the upstream extension of the N-terminus comprises the addition of the residues glycine-arginine-arginine in the (-3) to (-1) positions.
13. A method of improving the bleachability of wood pulp, the method comprising treating the pulp with a xylanase according to claim 1 for approximately 5 minutes to three hours, at a temperature between approximately 55°C to 75°C.
14. The method of claim 13 wherein the xylanase treatment is carried out at pH 7.5 to 9.0.
15. A modified xylanase according to claim 5 wherein the upstream extension of the N-terminus comprises the addition of the residues glycine-arginine-arginine in the (-3) to (-1) positions.
16. A modified xylanase according to claim 15 wherein the sequence of amino acids comprising amino acids 1-29 of *Trichoderma* xylanase II or the corresponding aligned sequence of another Family 11 xylanase is substituted by the corresponding aligned sequence of amino acids from xylanase from another Family 11 xylanase.
17. A modified xylanase according to claim 16 wherein the sequence of amino acids comprising amino acids 1-29 of

Trichoderma reesei xylanase II or the corresponding aligned sequence of another Family 11 xylanase are replaced with the corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A.

18. A method of modifying a Family 11 xylanase enzyme to improve any or all of the thermophilicity, alkalophilicity, and thermotolerance, the method consisting of one or more of the following three types of modifications:

- (1) Modification of amino acids 10, 27, or 29 of *Trichoderma reesei* xylanase II or the corresponding aligned amino acids of another Family 11 xylanase;
- (2) Substitution of one or more sequences of amino acids in the N-terminal region with that of corresponding aligned sequences of amino acids from another Family 11 xylanase to form a chimeric xylanase; and
- (3) Upstream extension of the N-terminus with the addition of up to 10 amino acids.

19. A method of improving the bleachability of wood pulp, the method comprising treating the pulp with a modified Family 11 xylanase according to claim 5 for 5 minutes to three hours at 55°C to 75°C.

20. The method of claim 19 wherein the xylanase treatment is carried out at pH 7.5 to 9.0.

21. A method of improving the bleachability of wood pulp, the method comprising treating the pulp with a modified Family 11 xylanase according to claim 2 for approximately 5 minutes to three hours, at a temperature between approximately 55°C to 75°C.

22. The method of claim 21 wherein the xylanase treatment is carried out at pH 7.5 to 9.0.

23. A method of improving the bleachability of wood pulp, the method comprising treating the pulp with a modified Family 11 xylanase according to claim 6 for approximately 5 minutes to three hours, at a temperature between approximately 55°C to 75°C.

24. The method of claim 23 wherein the xylanase treatment is carried out at pH 7.5 to 9.0.

25. A modified xylanase according to claim 1 wherein said xylanase is selected from the group consisting of NI-TX6, NI-TX10, NI-TX11, NI-TX12 or NI-TX13.

Figure 1

Abbreviations of species

Bp	<i>Bacillus pumilus</i>
Ca	<i>Clostridium acetobutylicum</i> P262 XynB
Cs	<i>Clostridium stercoarium</i> xynA
Rf	<i>Ruminococcus flavefaciens</i>
Tr2	<i>Trichoderma reesei</i> XYN II
Tv	<i>Trichoderma viride</i>
Th	<i>Trichoderma harzianum</i>
Sc	<i>Schizophyllum commune</i> Xylanase A
An	<i>Aspergillus niger</i> , var. <i>awamori</i>
At	<i>Aspergillus tubigensis</i>
Tr1	<i>Trichoderma reesei</i> XYN I
Ss	<i>Streptomyces</i> sp. 36a
SlB	<i>Streptomyces lividans</i> Xln B
SlC	<i>Streptomyces lividans</i> Xln C
Tf	<i>Thermomonospora fusca</i> TfxA
Bc	<i>Bacillus circulans</i>
Bs	<i>Bacillus subtilis</i>

Aligned amino acid sequences numbering from N-terminusFirst section

<u>Lowest Amino acid #</u>		<u>Highest amino acid #</u>	
Ca	23	S AFNTQAAP	31
Cs	1	G	1

Second Section

<u>Lowest amino acid #</u>		<u>Highest amino acid #</u>	
Bp	1	RTITNNEMGN HSGYDYELWK DYGNT-SMTL NNGGAFSAGW N--NIGNA	45
Ca	32	KTITSNEIGV NGGYDYELWK DYGNT-SMTL KNGGAFSCQW S--NIGNA	76
Cs	2	RIIYDNETGT HGGYDYELWK DYGNT-IMEL NDGGTFSCQW S--NIGNA	46
Rf	1	SAADQQTRGN VGGYDYEMWN QNGQGQASMN PGAGSFTCSW S--NIENF	46
Tr2	1	QTIQPGTGY NNGYFYSYWN DGHGGVTTYTN GPGGQFSVNW S--NSGNF	45
Tv	1	QTIQPGTGF NNGYFYSYWN DGHGGVTTYTN GPGGQFSVNW S--NSGNF	45
Th	1	QTIGPGTGY SNGYYSYWN DGHAGVTTYTN GGGGSFTVNW S--NSGNF	45
Sc	1	SGTPSSTGT DGGYYSYWN DGAGDATYQN NGGGSYTLTH SG--NNGNL	46
An	1	S AGINYVQNYN GNLGDFTY-D ESAGTFSMYH EDGVSSDF	37
AT	1	S AGINYVQNYN QNLGDFTY-D ESAGTFSMYH EDGVSSDF	37
Tr1	1	ASINYDQNYQ TGG-QVSY- PSNTGFSVNW N--TQDDF	34
Ss	1	ATTIT-NETGY D-GMYYSFWT DGGGSVSMTL NGGGSYSTRW T--NCGNF	45
SlB	1	DTVVTNQEGT NNGYYSFWT DSQGTVMNM GSGGQYSTSW R--NTGNF	47
SlC	1	ATTITNTQTGT D-GMYYSFWT DGGGSVSMTL NGGGSYSTOW T--NCGNF	46
Tf	1	AVTSNETGY HDGYFYSFWT DAPGTVSMEL GPGGNYSTSW R--NTGNF	45
Bc	1	ASTDYWQNW DGGGIVNAVN GSGGNYSVNW S--NTGNF	36
Bs	1	ASTDYWQNW DGGGIVNAVN GSGGNYSVNW S--NTGNF	36

Figure 1 (continued)

Third section

		<u>Lowest amino acid #</u>				<u>Highest amino acid #</u>	
Bp	46	LFRK-GKKD	ST-RTHHQLG	NISINYNASF	N-PSGNSYLC	VYGWTQSP	90
Ca	77	LFRK-GKKFN	DT-QTYKQLG	NISVNYNCNY	Q-PYGNSYLC	VYGWTSSP	121
Cs	47	LFRK-GRKFN	SD-KTYQELG	DIVVEYGCYD	N-PNGNSYLC	VYGWTRNF	91
Rf	47	LARM-GKNYD	SQKKNYKAFG	NIVLTYDVEY	T-PRGNSYMC	VYGWTRNP	92
Tr2	46	VGGK-GWQPG	TKNKV-----	---INFS-GS	YNPNGNSYLS	VYGWSRNP	83
Tv	46	VGGK-GWQPG	TKNKV-----	---INFS-GS	YNPNGNSYLS	VYGWSRNP	83
Th	46	VGGK-GWQPG	TKNKV-----	---INFS-GS	YNPNGNSYLS	VYGWSRNP	83
Sc	47	VGGK-GWNP	AASRS-----	---ISYS-GT	YQPNGNSYLS	VYGWTRSS	84
An	38	VVGL-GWTTG	SSNA-----	---ITYSAEY	SASGSSSYLA	VYGVVNYP	76
At	38	VVGLGGWTTG	SSNA-----	---ITYSAEY	SASGSASYLA	VYGVVNYP	76
Tr1	35	VVGW-GWTTG	SSAP-----	---INFGGSF	SVNSGTGLLS	VYGWSTNP	72
Ss	46	VAGK-GWANG	GR-RT-----	---VRYT-GW	FNPSGNGYGC	LYGWTSTNP	82
SlB	48	VAGK-GWANG	GR-RT-----	---VOYS-GS	FNPSGNAYLA	LYGWTSTNP	84
SlC	47	VAGK-GWSTG	DGN-----	---VRYN-GY	FNPSGNGYGC	LYGWTSTNP	82
Tf	46	VAGK-GWATG	GR-RT-----	---VTYS-AS	FNPSGNAYLT	LYGWTRNP	82
Bc	37	VVGK-GWTTG	SPFRT-----	---INYNAGV	WAPNGNGYLT	LYGWTRSP	75
Bs	37	VVGK-GWTTG	SPFRT-----	---INYNAGV	WAPNGNGYLT	LYGWTRSP	75

Fourth section

		<u>Lowest amino acid #</u>				<u>Highest amino acid #</u>	
Bp	91	LAEXYIVDSW	GTYS-PT--G	AYKGSFYADG	GTYDIYETTR	VNOPSIIIG	135
Ca	122	LVEXYVIDSW	GSWRPP--GG	TSKGTITVDG	GIYDIYETTR	INOPSIOG	167
Cs	92	LVEXYIVESW	GSWRPP--GA	TPKGTITQWMA	GTYEIYETTR	VNOPSIDG	138
Rf	93	LMEXYIVEGW	GDWRPPGNDG	EVKGTVSANG	NTYDIRKTMR	VNOPSIDG	140
Tr2	84	LIEXYIVENF	GTYN-PSTGA	TKLGEVTSQG	SVYDIYRTOR	VNOPSIIIG	130
Tv	84	LIEXYIVENF	GTYN-PSTGA	TKLGEVTSQG	SVYDIYRTOR	VNOPSIIIG	130
Th	84	LIEXYIVENF	GTYN-PSTGA	TKLGEVTSQG	SVYDIYRTOR	VNOPSIIIG	130
Sc	85	LIEXYIVESY	GSYD-PSSAA	SHKGSVTCNG	ATYDILSTWR	YNAPSIDG	131
An	77	GAEXYIVEDY	GDYN-PCSSA	TSLGTVYSDG	STYQVCTDTR	INEPSITG	123
At	77	QAEXYIVEDY	GDYN-PCSSA	TSLGTVYSDG	STYQVCTDTR	INEPSITG	123
Tr1	73	LVEXYIMEDN	HNY--PAQ-G	TVKGTVTSQG	ATYTIWENTR	VNEPSIOG	117
Ss	83	LVEXYIVDNW	GSYR-PT--G	ETRGTVHSDG	GTYDIYKTTR	YNAPVEVA	127
SlB	85	LVEXYIVDNW	GTYS-PT--G	EYKGTVTSQG	GTYDIYKTTR	VNKPEVEG	129
SlC	83	LVEXYIVDNW	GSYR-PT--G	TYKGTVSSDG	GTYDIYQTTR	YNAPVEVEG	127
Tf	83	LVEXYIVESW	GTYS-PT--G	TYMGTVTTDG	GTYDIYKTTR	YNAPSEIEG	127
Bc	76	LIEXYVVDWS	GTYS-PT--G	TYKGTVKSQG	GTYDIYTTTR	YNAPSIDG	120
Bs	76	LIEXYVVDWS	GTYS-PT--G	TYKGTVKSQG	GTYDIYTTTR	YNAPSIDG	120

Figure 1 (continued)

Fifth section

<u>Lowest amino acid #</u>		<u>Highest amino acid #</u>	
Bp	136	-IATEKQYWE VRQTKRTS--	-----GTVS VSAHFRKWES LGMPM-GK 173
Ca	168	-NTTFKQYWE VRRTKRTS--	-----GTIS VSKHFAAWES KGMPPL-GK 206
Cs	139	-TATEFQYWE VRTSKRTS--	-----GTIS VTEHFKOWER MGMRM-GK 177
Rf	141	-TATEFQYWE VRQTS GSANN	QTN YMKG TID VSKHFD A WSA AGLDMSGT 187
Tr2	131	-TATEFYQYWE VRNHR-S-S	-----GSVN TANHFNAWAQ OGLTL-GT 168
Tv	131	-TATEFYQYWE VRTHR-S-S	-----GSVN TANHFNAWAQ OGLTL-GT 168
Th	131	-TATEFYQYWE VRNHR-S-S	-----GSVN TANHFNAWAS HGLTL-GT 168
Sc	132	-TOTFEQFWE VRNPKKAPGG	SIS---GTVD VOCHFDAWKG LGMNLGSE 175
An	124	-TSTFTQYFE VRESTRTS--	-----GTVT VANHFNFHQAQ HGFNG-SD 162
At	124	-TSTFTQYFE VRESTRTS--	-----GTVT VANHFNFHAH HGFHN-SD 162
Tr1	118	-TATFNQYIS VRNSPR-T-S	-----GTVT VQNHFN-WAS LGLHLGQM 155
Ss	128	-PAAFDQYWE VRQSKVT--S	-----GTIT TGNHFD A WAR AGMNMGNF 166
SlB	130	TR-TFDQYWE VRQSKR-TG-	-----GTIT TGNHFD A WAR AGMPLGNF 168
SLC	128	TK-TFQYWE VRQSKVTS GS	-----GTIT TGNHFD A WAR AGMNMGQF 168
Tf	128	TR-TFDQYWE VRQSKRTS--	-----GTIT AGNHFD A WAR HGMHLGTH 166
Bc	121	DRTTFTQYWE VRQSKRPTGS	N-----ATIT FTNHVNAWKS HGMNLGSN 163
Bs	121	DRTTFTQYWE VRQSKRPTGS	N-----ATIT FSNHVNAWKS HGMNLGSN 163

Sixth section

<u>Lowest amino acid #</u>		<u>Highest amino acid #</u>	
Bp	174	MYETAFTVEG YQSSGSANVM	TNQLFIGN 201
Ca	207	MHETAFNIEG YQSSGKADVN	SMSINIGK 233
Cs	178	MYEVALTVEG YQSSGYANVY	KNEIRIGANP....
Rf	188	LYEVSLNIEG YRNGSANVK	SVSV 211
Tr2	169	MDYQIVAVEG YFSSGSASI-	TVS 190
Tv	169	MDYQIVAVEG YFSSGSASI-	TVS 190
Th	169	MDYQIVAVEG YFSSGSASI-	TVS 190
Sc	176	HNYQIVATEG YQSSGTATI-	TVT 197
An	163	FNYQVMAVEA WSGAGSASV-	TISS 184
At	163	FNYQVMAVEA WSGAGSAAV-	TISS 184
Tr1	156	MNYQVMAVEG WGGSGSASQ-	SVSN 178
Ss	167	RYYMINATEG YQSSGSSTI-	TVSG 189
SlB	169	SYMINATEG YQSSGTSSI-	NVGG.....
SLC	169	RYYMINATEG YQSSGSSNI-	TVSG 191
Tf	167	D-YMIMATEG YQSSGSSNVT	LGTS.....
Bc	164	WAYQVMATEG YQSSGSSNV-	TVW 185
Bs	164	WAYQVMATEG YQSSGSSNV-	TVW 185

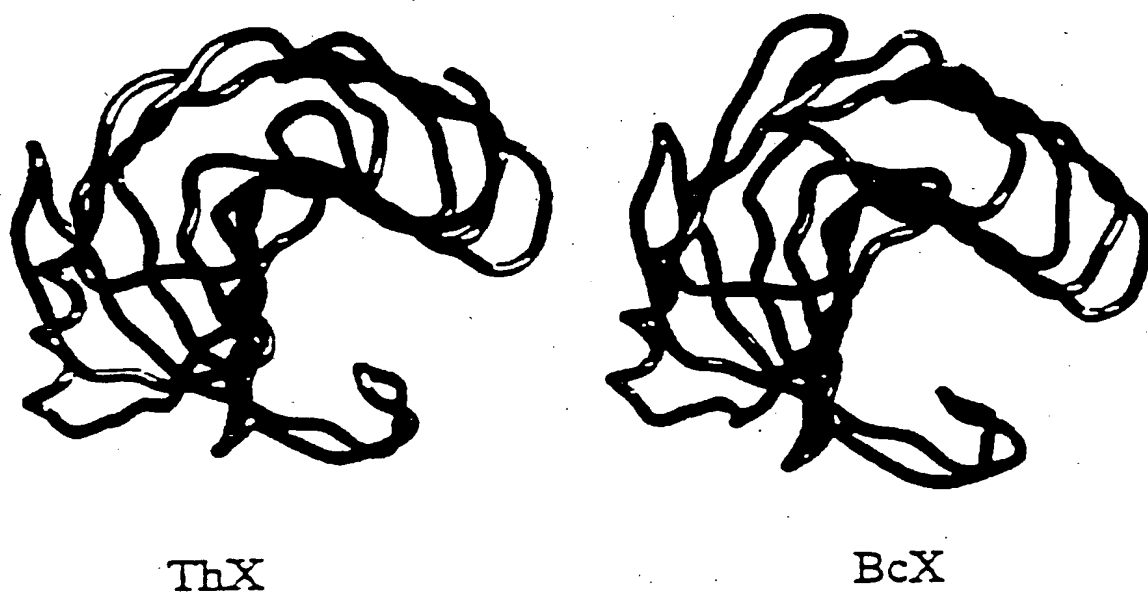


Figure 2

																XyTV-1							
A	S	I	G	P	G	T	G	P	N	N	G	Y	F	Y	S								
CT	AGC	ATA	GGA	CCA	GGA	ACC	GGT	TTC	AAC	AAC	GGT	TAC	TTT	TAC	AGC								
G		TAT	CCT	GGT	CCT	TGG CCA		AAG	TTG	TTG	CCA	ATG	AAA	ATG	TCG								
NheI								AgeI		XyTV-8													
																XyTV-2							
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32								
Y	W	N	D	G	H	G	G	V	T	Y	T	N	G	P	G								
TAT	TGG	AAC	GAT	GGC	CAT	GGT	GGT	GTT	ACC	TAT	ACA	AAC	GGG	CCC	GGA								
ATA	ACC	TTG	CTA	CCG	GTA	CCA	CCA	CAA	TGG	ATA	TGT	TTG	CCC	GGG	CCT								
						NcoI		XyTV-7						ApaI									
																XyTV-3							
33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48								
G	Q	F	S	V	N	W	S	N	S	G	N	F	V	G	G								
GGC	CAA	TTT	AGC	GTC	AAT	TGG	TCT	AAC	TCC	GGA	AAC	TTT	GTA	GGT	GGA								
CCG	GTT	AAA	TCG	CAG	TTA	ACC	AGA	TTG	AGG	CCT	TTG	AAG	CAT	CCA	CCT								
						MunI		BspEI															
																XyTV-6							
49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64								
K	G	W	Q	P	G	T	K	N	K	V	I	N	F	S	G								
AAA	GGT	TGG	CAA	CCC	GGG	ACC	AAA	AAT	AAG	GTG	ATC	AAC	TTC	TCT	GGA								
TTT	CCA	ACC	GTT	GGG	CCC	TGG	TTT	TTA	TTC	CAC	TAG	TTG	AAG	AGA	CCT								
						XmaI																	
																XyTV-4							
65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80								
S	Y	N	P	N	G	N	S	Y	L	S	V	Y	G	W	S								
TCT	TAT	AAT	CCG	AAT	GGG	AAT	TCA	TAC	TTA	AGC	GTC	TAT	GGC	TGG	TCT								
AGA	ATA	TTA	GGC	TTA	CCC	TTA	AGT	ATG	AAT	TCG	CAG	ATA	CCG	ACC	AGA								
						EcoRI		AflII															
																XyTV-5							
81	82	83	84	85	86	87	88	89	90	91	92	93	94	95									
R	N	P	L	I	E	Y	Y	I	V	E	N	F	G	T									
AGA	AAC	CCA	CTG	ATT	GAA	TAT	TAC	ATT	GTC	GAA	AAT	TTC	GGT	AC									
TCT	TTG	GGT	GAC	TAA	CTT	ATA	ATG	TAA	CAG	CTT	TTA	AAG	C										
XbaI														KpnI									

XyTV-101															
	92	93	94	95	96	97	98	99	100	101	102	103	104	105	
V	D	N	F	G	T	Y	N	P	S	T	G	A	T	K	L
TC	GAC	AAT	TTC	GGT	ACC	TAC	AAT	CCG	AGT	ACC	GGC	GCC	ACA	AAA	TTA
	G	TTA	AAG	<u>CCA TGG</u>	ATG	TTA	GGC	TCA	TGG	<u>CCG CGG</u>	TGT	TTT	AAT		
SallI				KpnI					XyTV-110		KasI/NarI				
XyTV-102															
106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121
G	E	V	T	S	D	G	S	V	Y	D	I	Y	R	T	Q
GGC	GAA	GTC	<u>ACT</u>	AGT	GAT	GGA	TCC	GTA	TAT	GAT	ATC	TAC	CGT	ACC	CAA
CCG	CTT	CAG	<u>TGA TCA</u>	CTA	<u>CCT AGG</u>	CAT	ATA	CTA	TAG	ATG	GCA	TGG	GTT		
			SpeI			BamHI							XyTV-109		
XyTV-103															
122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137
R	V	N	Q	P	S	I	I	G	T	A	T	F	Y	Q	Y
CGC	GTT	AAT	CAG	CCA	<u>TCS</u>	ATC	ATT	GGA	ACC	<u>GCC</u>	ACC	TTT	TAT	CAG	TAC
<u>GCG</u>	<u>CAA</u>	TTA	GTC	GGT	<u>AGC</u>	<u>TAG</u>	TAA	CCT	TGG	<u>CGG</u>	<u>TGG</u>	AAA	ATA	GTC	ATG
MluI					claiI										
138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153
W	S	V	R	R	T	H	R	S	S	G	S	V	N	T	A
TGG	AGT	GTT	AGA	CGT	ACG	CAT	CGG	AGC	<u>TCC</u>	<u>GGT</u>	<u>TCG</u>	<u>GTT</u>	<u>AAT</u>	<u>ACT</u>	<u>GCG</u>
ACC	TCA	CAA	TCT	<u>GCA</u>	<u>TGC</u>	GTA	<u>GCC</u>	<u>TCG</u>	<u>AGG</u>	CCA	AGC	CAA	TTA	TGA	CGC
XyTV-108					BsiWI				SacI						
XyTV-104															
154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169
N	H	F	N	A	W	A	Q	Q	G	L	T	L	G	T	M
AAT	CAC	TTT	AAT	GCA	TGG	GCA	CAG	CAA	GGG	TTA	ACC	CTA	GGT	ACA	ATG
TTA	GTG	AAA	<u>TTA</u>	<u>CGT</u>	ACC	CGT	GTC	GTT	CCC	AAT	<u>TGG</u>	<u>GAT</u>	<u>CCA</u>	TGT	TAC
			NsiI										AvrII		
XyTV-105															
170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185
D	Y	Q	I	V	A	V	E	G	Y	F	S	S	G	S	A
<u>GAT</u>	<u>TAT</u>	<u>CAA</u>	<u>ATC</u>	<u>GTA</u>	<u>GCG</u>	<u>GTG</u>	<u>GAA</u>	<u>GGC</u>	<u>TAC</u>	<u>TTC</u>	<u>TCG</u>	<u>AGT</u>	<u>GGT</u>	<u>TCC</u>	<u>GCT</u>
CTA	ATA	GTT	TAG	CAT	CGC	CAC	CTT	CCG	ATG	AAG	<u>AGC</u>	<u>TCA</u>	CCA	AGG	CGA
						XyTV-106					XhoI				
186	187	188	189	190											
S	I	T	V	S											
AGT	ATT	ACA	GTG	AGC	TAA	A									
TCA	TAA	TGT	CAC	TCG	ATT	<u>TCT</u>	AG								
						BglIII									

FIGURE 3 CONT'D

XY-11															
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A	S	T	D	Y	W	Q	N	W	T	D	G	G	G	I	V
GCT	AGC	ACA	GAT	TAC	TGG	CAA	AAC	TGG	ACA	GAC	GGT	GGC	GGT	ATC	GTT
<u>CGA</u>	<u>TCG</u>	TGT	CTA	ATG	ACC	GTT	TTG	ACC	TGT	CTG	CCA	CCG	CCA	TAG	CAA
NheI															
XY-16															
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
N	A	V	N	G	S	G	G	N	Y	S	V	N	W	S	N
AAT	GCC	GTG	AAC	GGC	TCC	GGA	GGC	AAC	TAC	AGC	GTG	AAT	TGG	TCT	AAT
TTA	CGG	CAC	TTG	CCG	<u>AGG</u>	<u>CCT</u>	CCG	TTG	ATG	TCG	CAC	TTA	ACC	AGA	TTA
BspEI															
XY-12															
33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
T	G	N	F	V	V	G	K	G	W	T	T	G	S	P	F
ACT	GGG	AAC	TTC	GTA	GTC	GGA	AAA	GGT	TGG	ACG	ACA	GGA	TCC	CCG	TTC
TGA	CCC	TTG	AAG	CAT	CAG	CCT	TTT	CCA	ACC	TGC	TGT	<u>CCT</u>	<u>AGG</u>	GGC	AAG
BamHI															
XY-15															
XY-21															
49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
R	T	I	N	Y	N	A	G	V	W	A	P	N	G	N	G
CCT	ACG	ATC	AAC	TAC	AAC	GCT	GGC	GTT	TGG	GCC	CCG	AAT	GGT	AAC	GGT
<u>GCA</u>	<u>TGC</u>	TAG	TTG	ATG	TTG	CGA	CCG	CAA	<u>ACC</u>	<u>CGG</u>	<u>GGC</u>	TTA	CCA	TTG	CCA
SphI															
XY-14a															
XY-22															
65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
Y	L	T	L	Y	G	W	T	R	S	P	L	I	E	Y	Y
TAC	CTG	ACA	CTG	TAT	GGC	TGG	ACG	CGT	TCG	CCA	CTG	ATT	<u>GAA</u>	<u>TAT</u>	<u>TAC</u>
ATG	GAC	TGT	GAC	ATA	CCG	ACC	<u>TGC</u>	<u>GCA</u>	AGC	GGT	GAC	TAA	CTT	ATA	ATG
MluI															
XY-24															
XY-23															
81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
V	V	D	S	W	G	T	Y	R	P	T	G	T	Y	K	G
GTT	GTC	GAC	TCT	TGG	GGA	ACG	TAC	CGT	CCG	ACT	GGA	ACC	TAC	AAA	GGC
CAA	<u>CAG</u>	<u>CTG</u>	AGA	ACC	CCT	TGC	ATG	GCA	GGC	TGA	CCT	TGG	ATG	TTT	CCG
SalI															
XY-3															
97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
T	V	K	S	D	G	G	T	Y	D	I	Y	T	T	T	R
ACA	GTC	AAA	AGC	GAT	GGT	GGT	ACC	<u>TAT</u>	GAC	ATC	TAC	ACC	ACC	ACA	AGA
TGT	CAG	TTT	TCG	CTA	CCA	<u>CCA</u>	<u>TGG</u>	ATA	CTG	TAG	ATG	TGG	TGG	TGT	TCT
KpnI															
XY-6															

FIGURE 4

			XY-1										
177	178	179	180	181	182	183	184	185					
S	G	S	S	N	V	T	V	W					
<u>TCT</u>	<u>GGT</u>	<u>TCT</u>	<u>TCC</u>	AAC	GTT	ACA	GTG	TGG	TAA	AGA	TCT	TGA	AGC TTGGGACGT
<u>AGA</u>	<u>CCA</u>	<u>AGA</u>	<u>AGG</u>	TTG	CAA	TGT	CAC	ACC	ATT	<u>TCT</u>	AGA	ACT TCG AACCC	
					XY-4a					BglIII	HindIII	AatII	

74

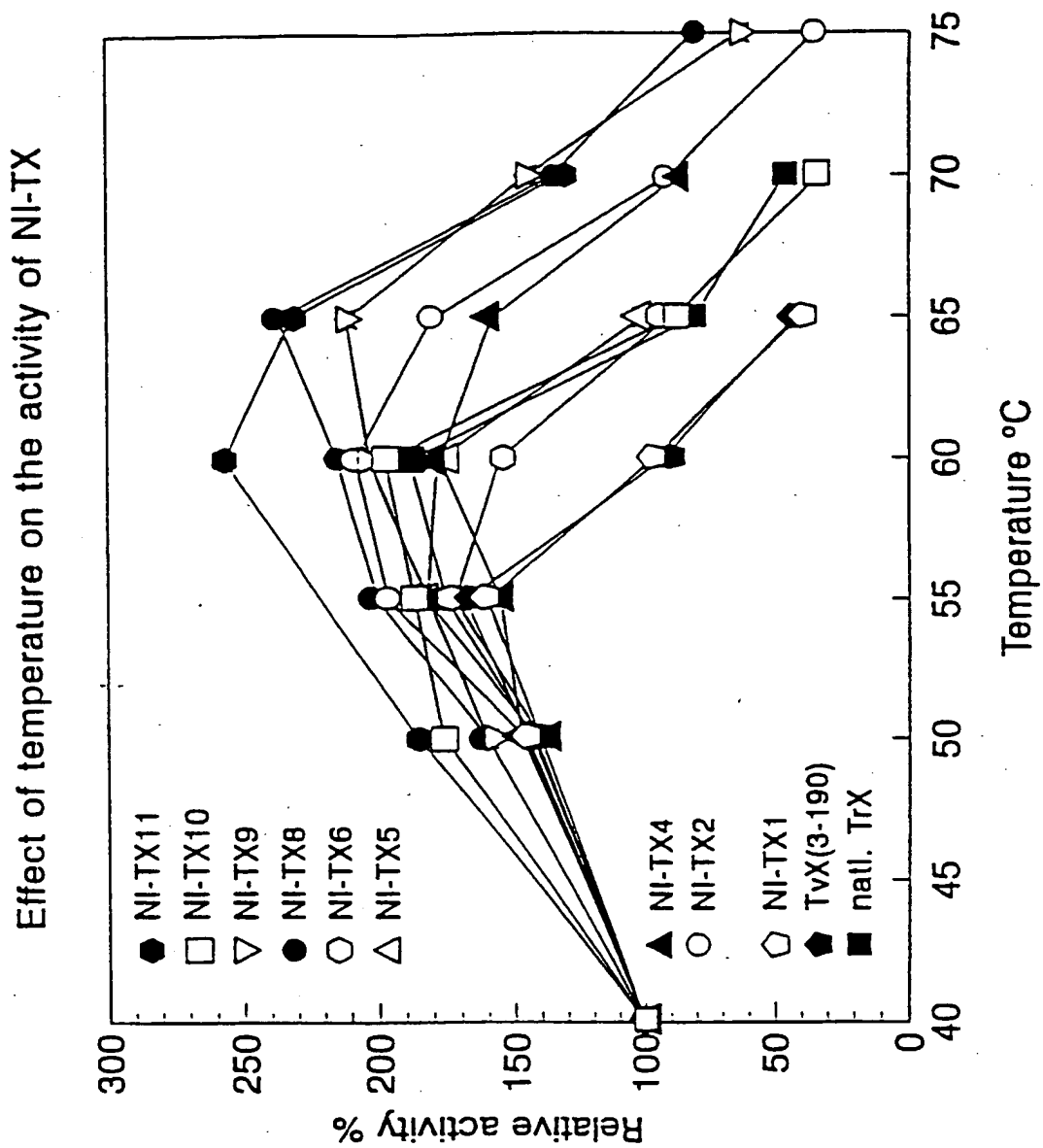


FIGURE 5

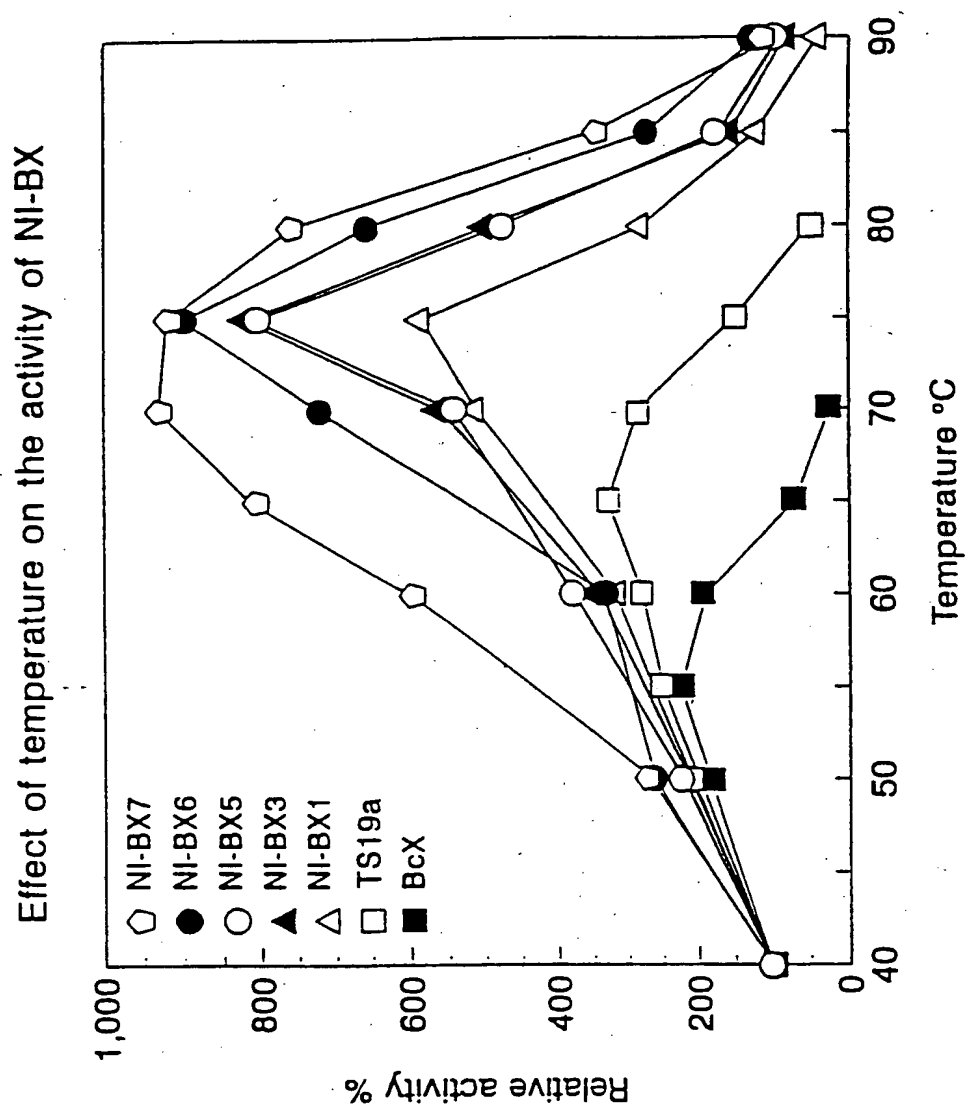


FIGURE 6

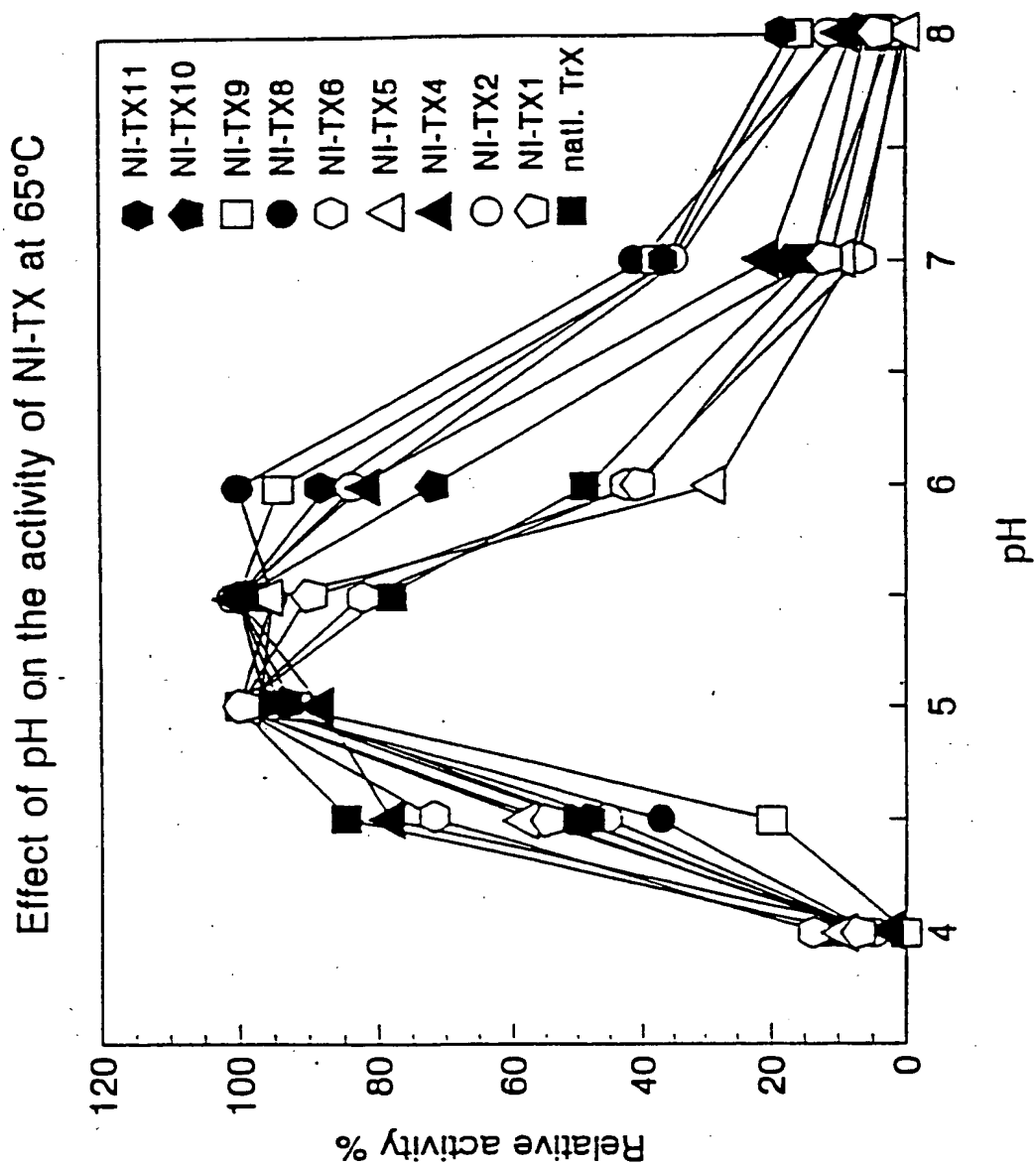


FIGURE 7

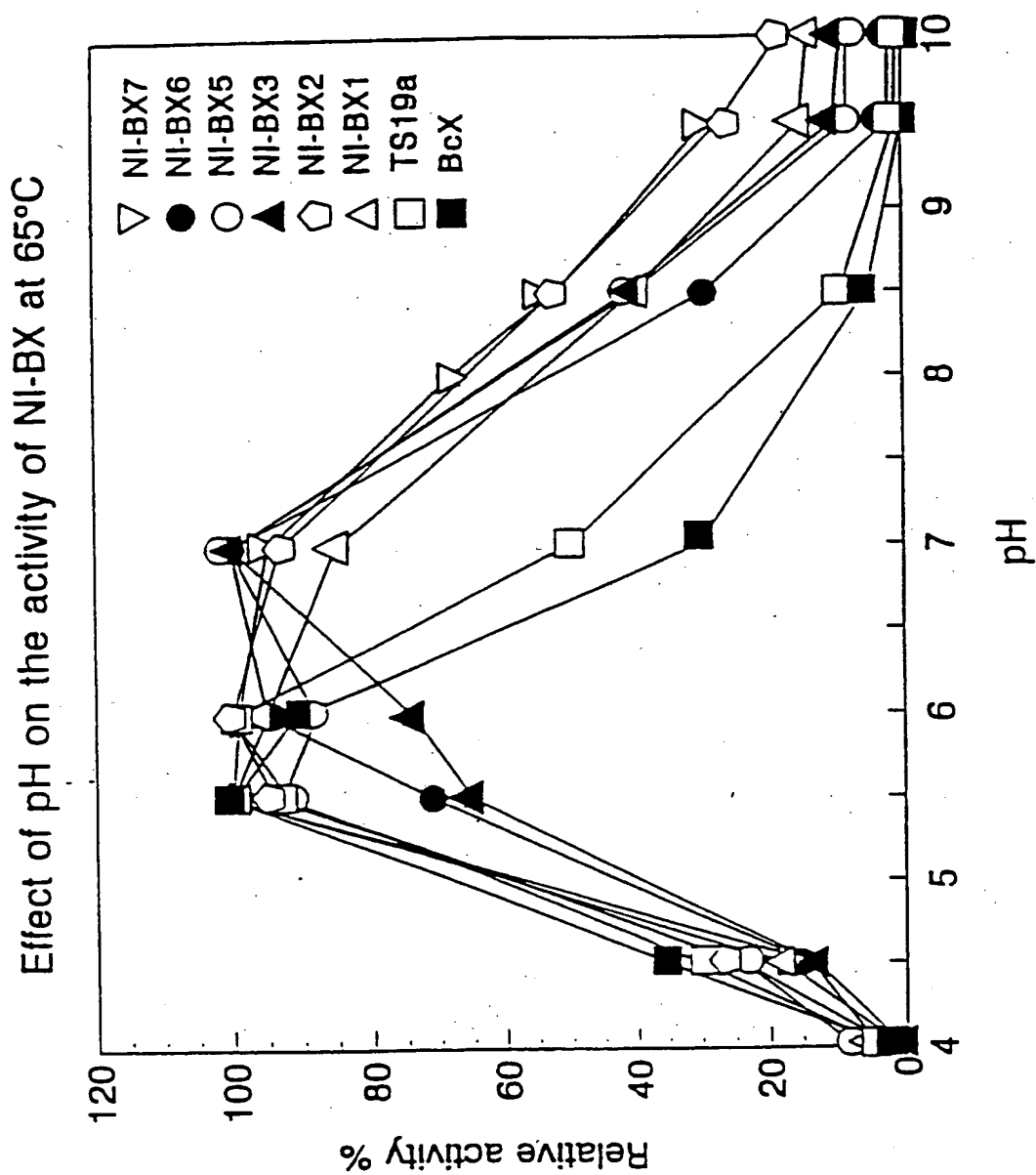


FIGURE 8

Effect of pH on the activity of NI-BX at 50°C

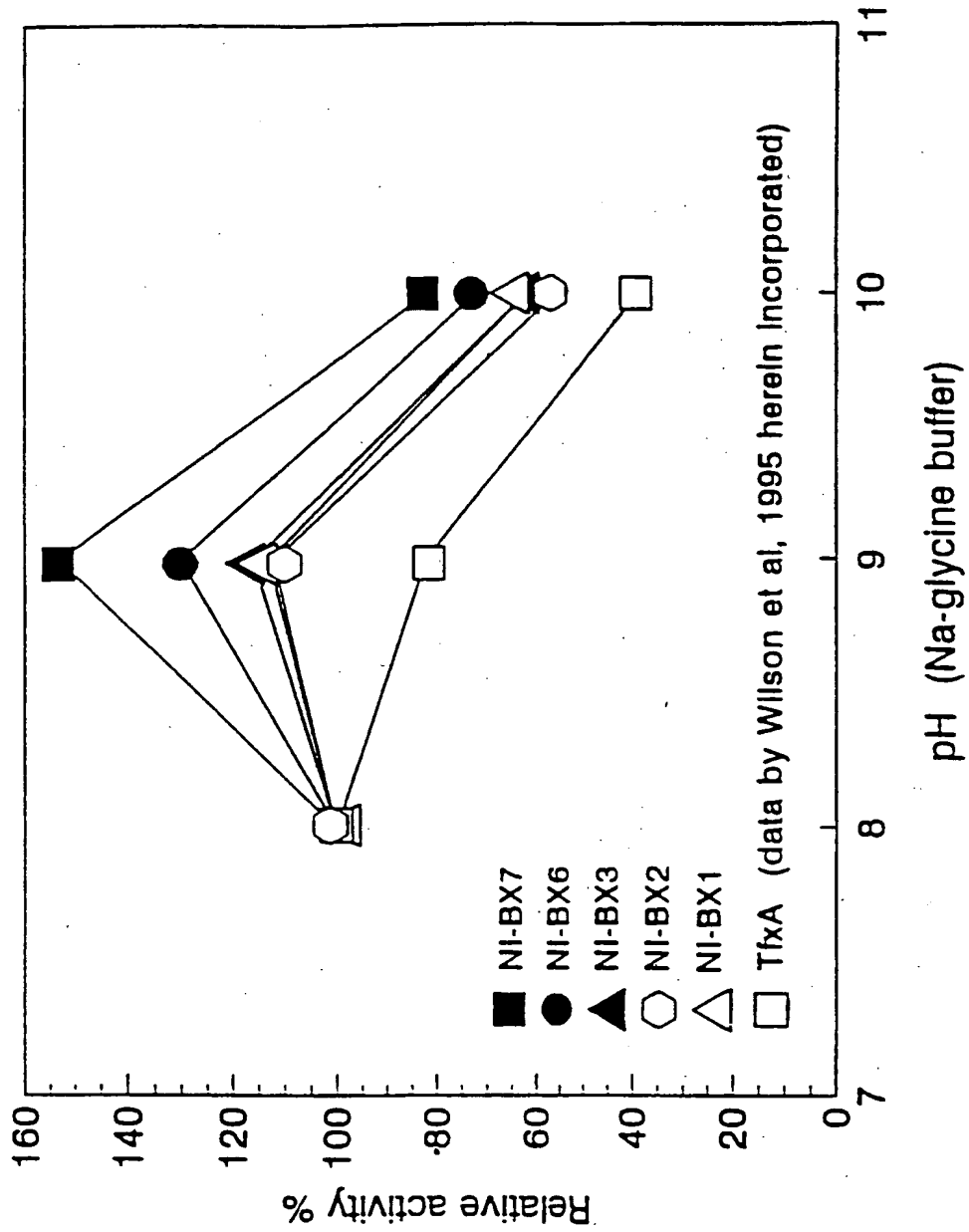


FIGURE 9

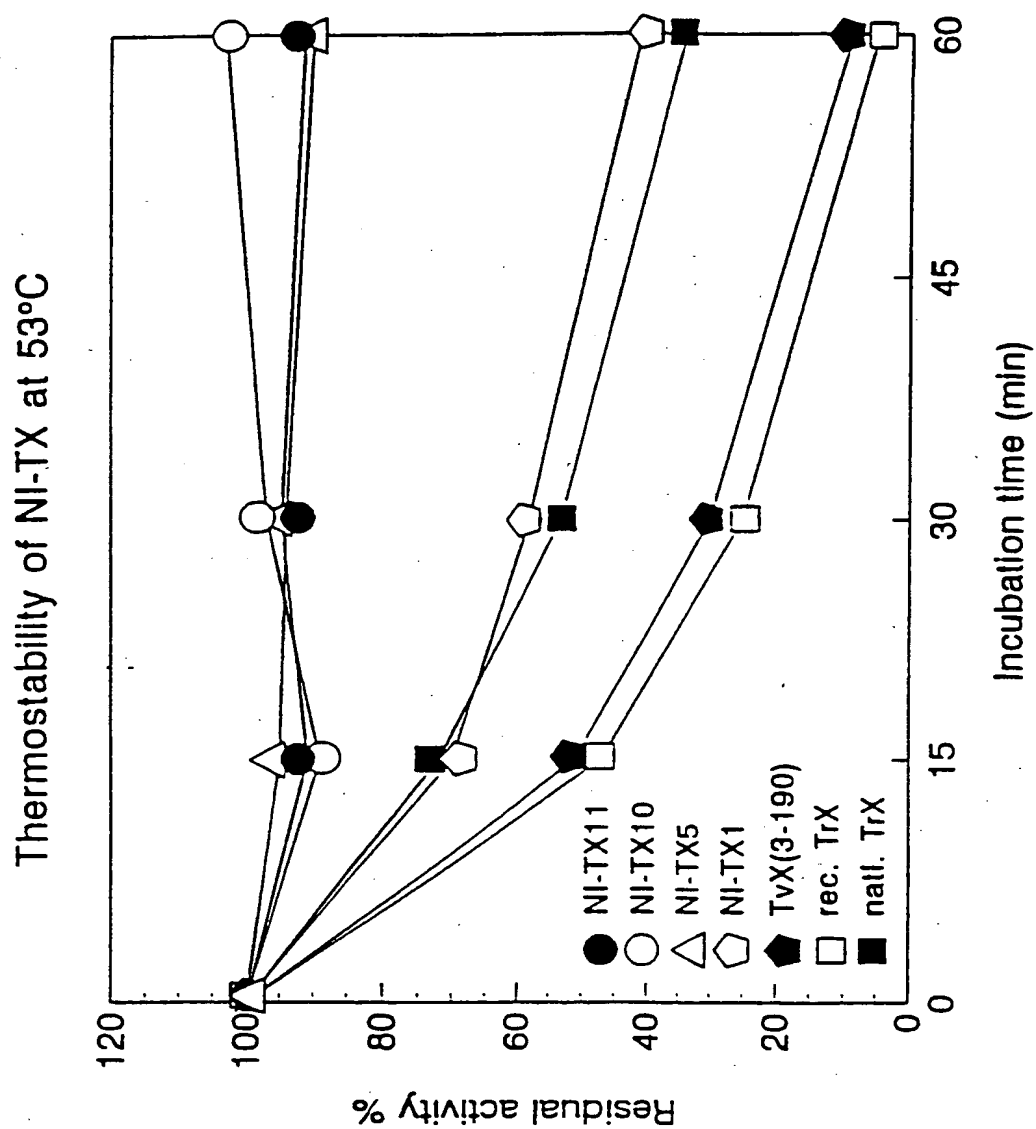


FIGURE 10

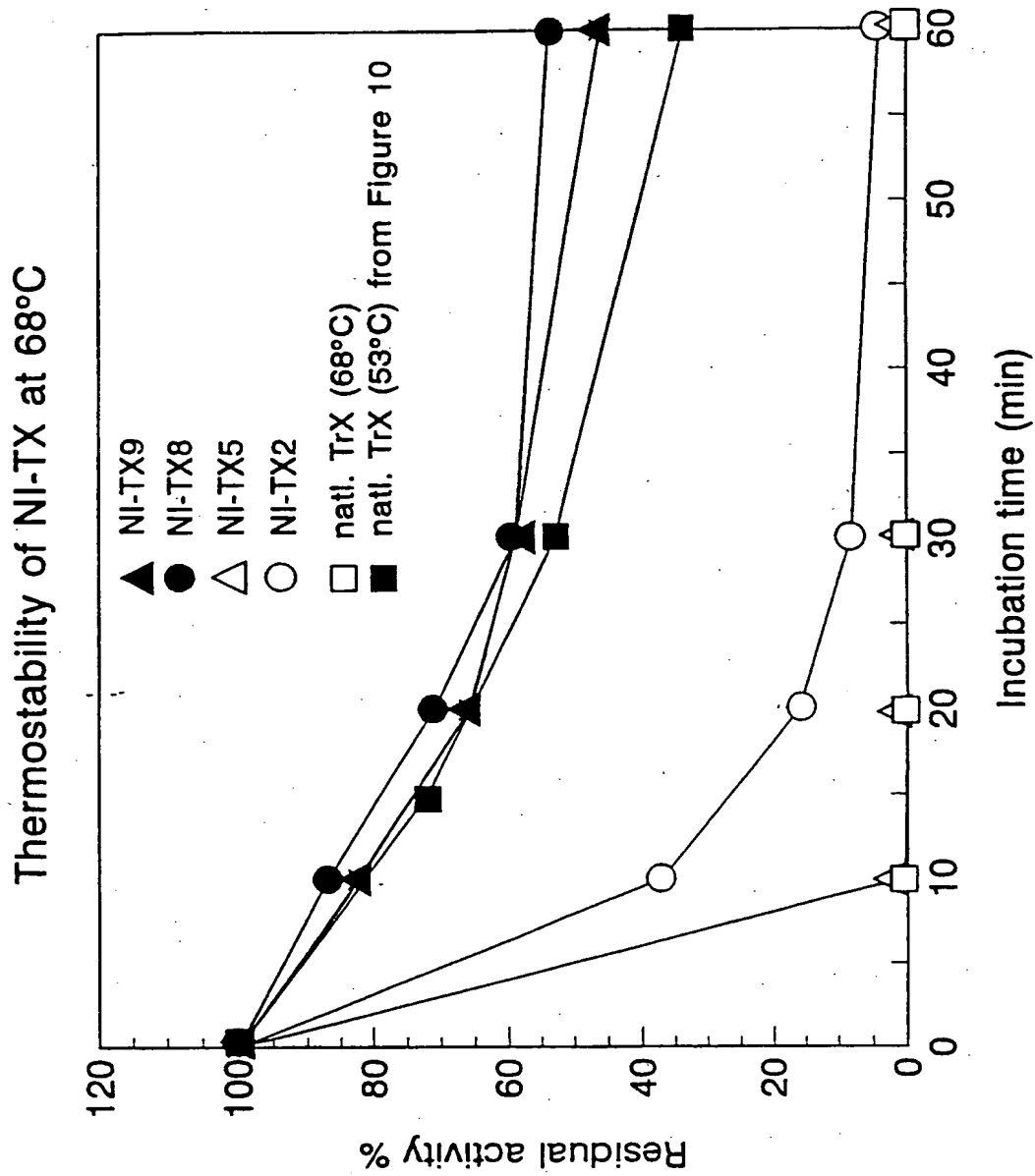


FIGURE 11

Thermostability of NI-BX at 70°C

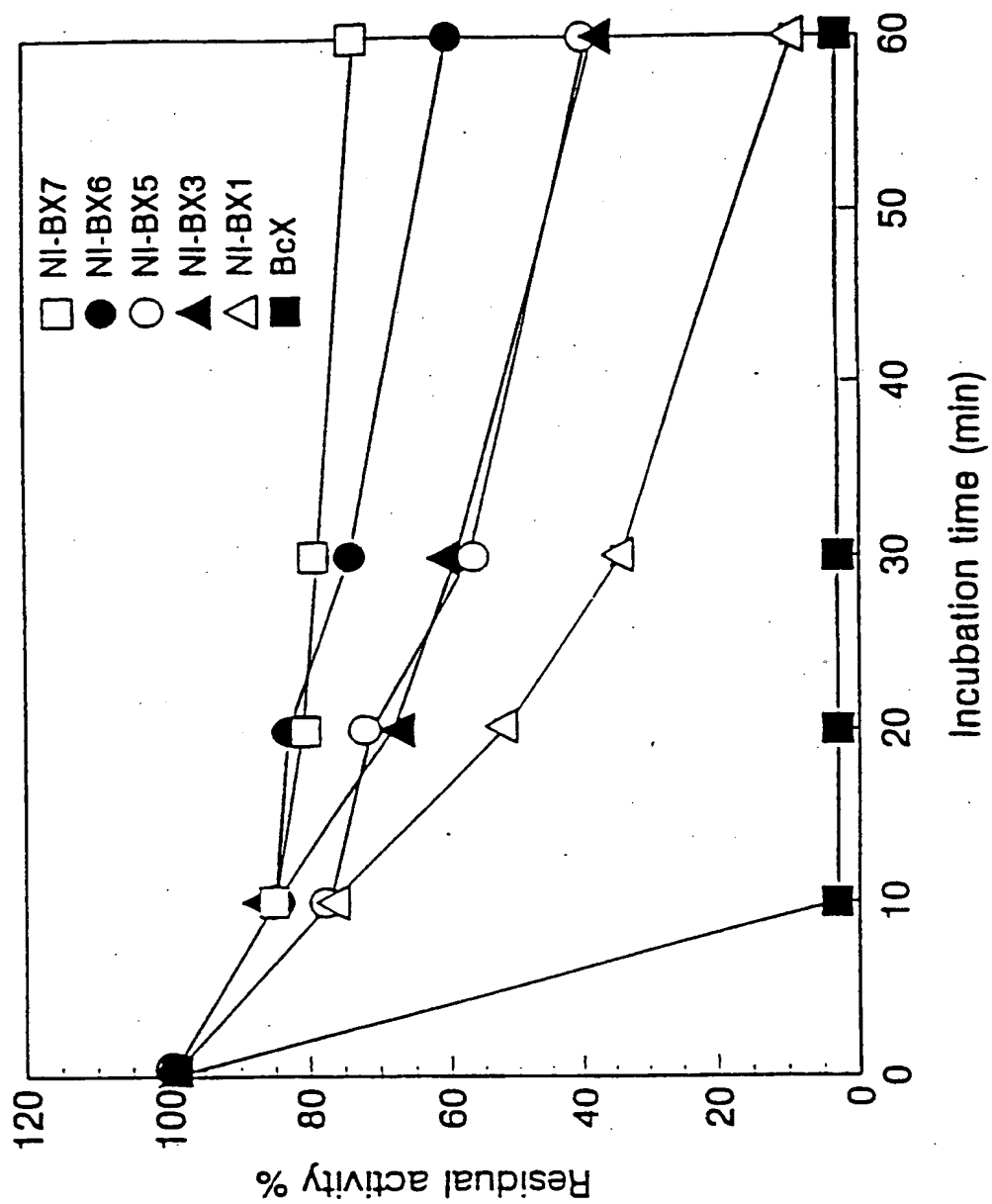


FIGURE 12

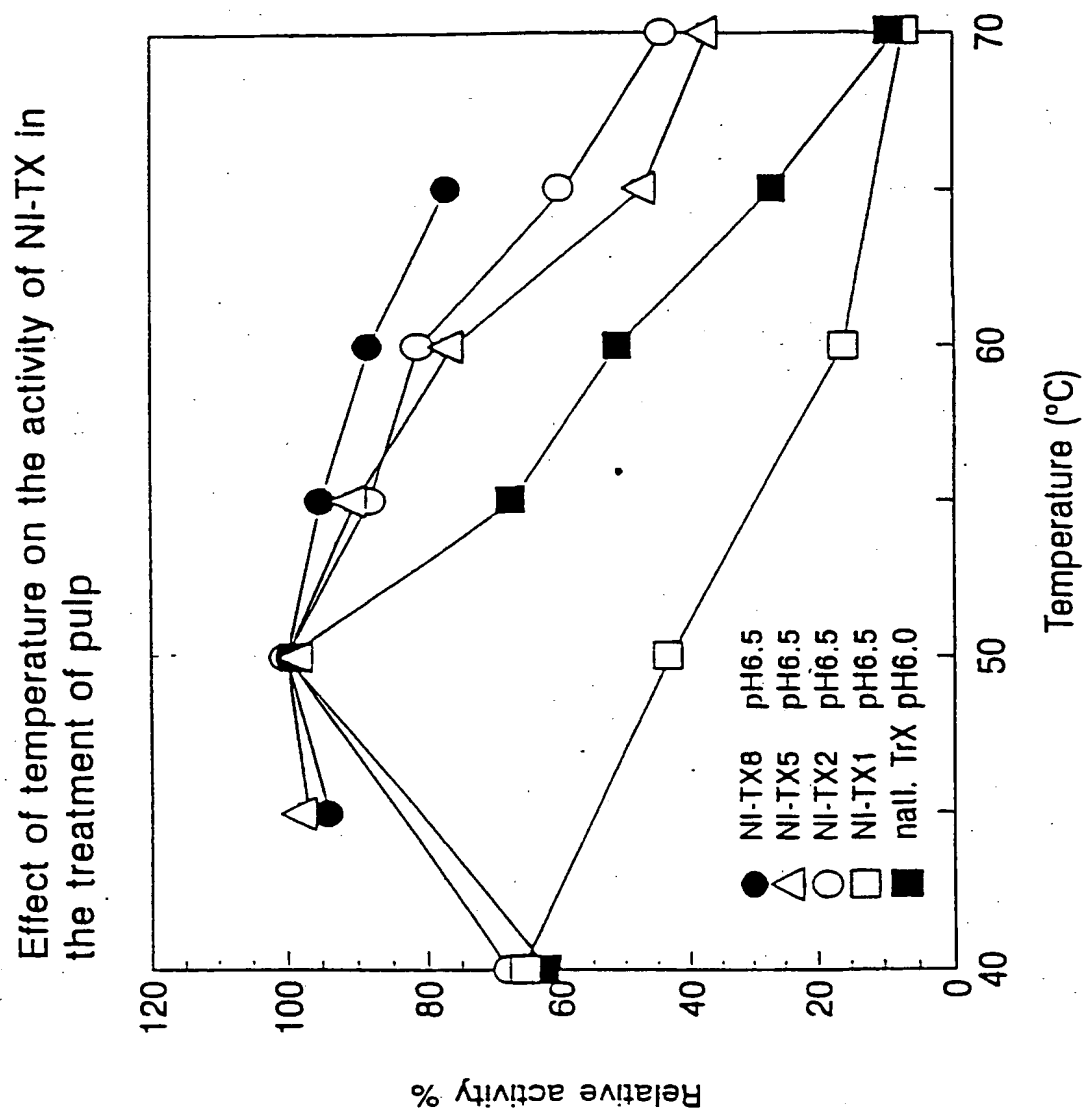


FIGURE 13

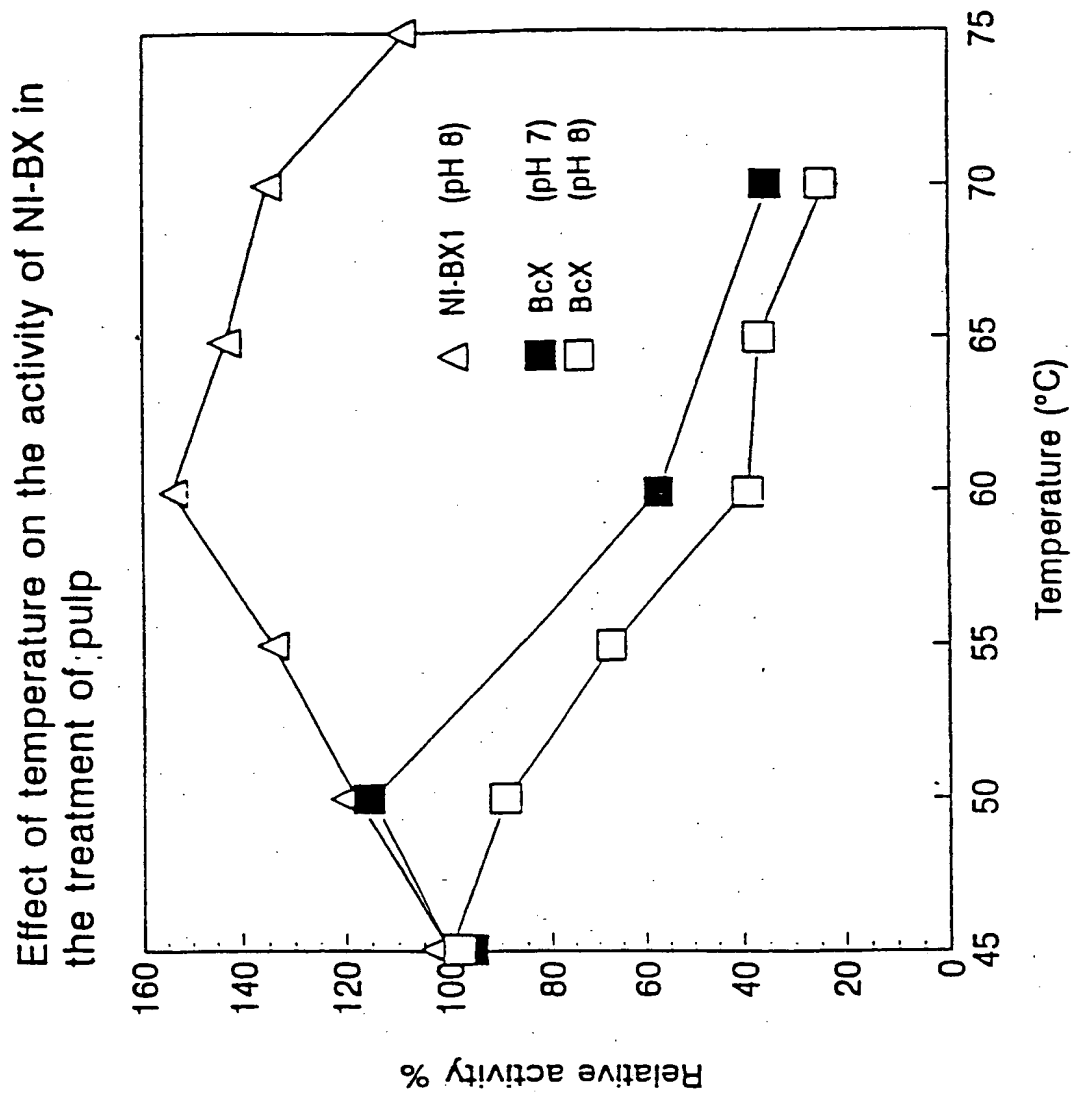


FIGURE 14

(19)



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(54) **Modification of xylanase to improve thermophilicity, alkophilicity and thermostability**

(57) Producing a xylanase enzyme of superior performance in the bleaching of pulp. More specifically, a modified xylanase of Family 11 that shows improved thermophilicity, alkalophilicity, and thermostability as compared to the natural xylanase. The modified xylanases contain any of three types of modifications: (1) changing amino acids 10, 27, and 29 of *Trichoderma reesei* xylanase II or the corresponding amino acids of another Family 11 xylanase, where these amino acids are changed to histidine, methionine, and leucine, respectively; (2) substitution of amino acids in the N-terminal region with amino acids from another xylanase enzyme. In a preferred embodiment, substitution of the natural *Bacillus circulans* or *Trichoderma reesei* xylanase with a short sequence of amino acids from *Thermomonospora fusca* xylanase yielded chimeric xylanases with higher thermophilicity and alkalophilicity; (3) an extension upstream of the N-terminus of up to 10 amino acids. In a preferred embodiment, extension of the N-terminus of the xylanase with the tripeptide glycine-arginine-arginine improved its performance.

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European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
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Place of search THE HAGUE		Date of completion of the search 24 July 1998	Examiner Hix, R
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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Application Number
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Application Number
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<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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